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This is a request for filing a (X) continuation application under 37 CFR 1.53(b), of pending prior application Serial No. 08/864,955 filed on May 29, 1997, of:

David Beach and Konstantin Galaktionov; Entitled: Novel cdc25 Genes, Encoded Products and Uses Thereof

Enclosed are:

96 page(s) of specification
4 page(s) of claims
1 page(s) of abstract
25 sheet(s) of drawing
5 page(s) of executed declaration and power of attorney

	CLAIMS	NO. FILED	NO. EXTRA	RATE	CALCULATIONS
	TOTAL CLAIMS (37 CFR 1.16(c))	-20=		x $22.00=	$
	INDEPENDENT CLAIMS (37 CFR 1.16(b))	-3=		x $82.00=	
	MULTIPLE DEPENDENT CLAIMS (if applicable) (37 CFR 1.16(d))			+ $270.00=	
				BASIC FEE (37 CFR 1.16(a))	+ $790.00
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By: James T Olesen
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Novel Human cdc25 Genes, Encoded Products and Uses Thereof

Related Applications

This application is a continuation-in-part of U.S.S.N. 08/379,685 filed 26 January 1995, which is a continuation-in-part of U.S.S.N. 08/124,569, filed 20 September 1993, which is a continuation-in-part of U.S.S.N. 07/793,601, filed 18 November 1991, and is a continuation-in-part of U.S.S.N. 08/189,206, filed 31 January 1994, which is a continuation-in-part of U.S.S.N. 07/878,640, filed 5 May 1992, and is a continuation-in-part of U.S.S.N. 07/793,601, filed 18 November 1991, the specification and claims of which are incorporated by reference herein.

Background of the Invention

In eukaryotic cells, mitosis is initiated following the activation of a protein kinase known as "M-phase promoting factor" (MPF; also known as the H-phase specific histone kinase, or more simply as the H-phase kinase). This kinase consists of at least three subunits: the catalytic subunit (cdc2), a regulatory subunit (cyclin B) and a low molecular weight subunit (p13-Suc1) (Brizuela, L. et al., EMBO J. 6:3507-3514 (1987); Dunphy, W. et al., Cell 54:423-431 (1988); Gautier, J. et al., Cell 54:433-439 (1988); Arion, D. et al., Cell 55:371-378 (1988); Draetta, G. et al., Cell 56:829-838 (1989); Booher, R. et al., Cell 58:485-497 (1989); Labbe, J-C. et al., EMBO J. 8:3053-3058 (1989); Meijer, L. et al., EMBO J. 8:2275-2282 (1989); Gautier, J. et al., Cell 60:487-494 (1990); Gautier, J. and J. Maller, EMBO J. 10:177-182 (1991)). cdc2 and related kinases also associate with other cyclins (Giordana, A. et al., Cell 58:981-990 (1989); Draetta, G. et al., Cell 56:829-838 (1989); Richardson, H.E. et al., Cell 59:1127-1133 (1989)), and comprise a family of related enzymes that act at various stages of the division cycle (Paris, J. et al., Proc. Natl. Acad. Sci. USA 88:1039-1043 (1990); Elledge, S.J. and M.R. Spottswood, EMBO J. 10:2653-2659 (1991); Tsai, L-H. et al., Nature 353:174-177 (1991)).

The cdc2/cyclin B enzyme is subject to multiple levels of control. Among these, the regulation of the catalytic subunit by tyrosine phosphorylation is the best understood. In a variety of eukaryotic cell types, cdc2 is one of the most heavily tyrosine phosphorylated

proteins (Draetta, G. et al., Nature 336:738-744 (1988); Dunphy, W.G. and J.W. Newport, Cell 58:181-431 (1989); Morla, A.O. et al., Cell 58:193-203 (1989)). Phosphorylation of the tyrosine 15 and also threonine 14 residues of cdc2 is regulated, in part, by the accumulation of cyclin above a threshold level at which association with cdc2 occurs (Solomon, M.J. et al., Cell 63:1013-1024 (1990)). Tyrosine phosphorylation inhibits the cdc2/cyclin B enzyme, and tyrosine dephosphorylation, which occurs at the onset of mitosis, directly activates the pre-MPF complex (Gautier J. et al., Nature 339:626-629 (1989); Labbe, J.C. et al., EMBO J. 8:3053-3058 (1989); Morla, A.O. et al., Cell 58:193-203 (1989); Dunphy, W.G. and J.W. Newport, Cell 58:181-431 (1989); Morla, A.O. et al., Cell 58:193-203 (1989); Gould, K. and P. Nurse, Nature 342:39-45 (1989); Jessus, C. et al., FEBS LETTERS 266:4-8 (1990)).

Given the role of cdc2 dephosphorylation in activation of MPF, there is much interest in the regulation of the cdc2 phosphatase. Genetic studies in fission yeast have established that the cdc25 gene function is essential for the initiation of mitosis (Nurse, P. et al., Mol. Gen. Genet. 146:167-178 (1976). The cdc25 gene product serves as a rate-determining activator of the cdc2 protein kinase (Russell, P. and P. Nurse, Cell 45:145-153 (1986); Ducommun, B. et al., Biochem. Biophys. Res. Common. 167:301-309 (1990); Moreno, S. et al., Nature 344:549-552 (1990)). Moreover, the mutant cdc2-F15, whose product cannot be phosphorylated on tyrosine, bypasses the requirement for cdc25 protein function (Gould, K. and P. Nurse, Nature 342:39-45 (1989)). Additional work has suggested that cdc25 is the cdc2 phosphatase. (Kumagai, A. and W.G. Dunphy, Cell 64:903-914 (1991); Strausfeld, U. et al., Nature 351:242-245 (1991)) and that cdc25 is the cdc2 phosphatase which dephosphorylates tyrosine and

possibly threonine residues on $p34^{cdc2}$ and regulates MPF activation. (Dunphy, W.G. and A. Kumagai, Cell 67:189-196 (1991); Gautier, J. et al., Cell 67:197-211 (1991)).

The universal intracellular factor MPF triggers the G2/M transition of the cell cycle in all organisms. In late G2, it is present as an inactive complex of tyrosine-phosphorylated $p34^{cdc2}$ and unphosphorylated cyclin B^{cdc13}. In M phase, its activation as an active MPF displaying histone H1 kinase activity originates from the specific tyrosine dephosphorylation of the $p34^{cdc2}$ subunit by the tyrosine phosphatase $p80^{cdc25}$. Little is known about the signals which control or determine timing of MPF activation and entry into mitosis or about ways in which those signals can be blocked or enhanced, resulting in inhibition or facilitation of entry into mitosis.

Because the signals that control dephosphorylation of cdc2 on tyrosine and threonine play a key role in controlling timing of MPF activation and entry into mitosis, there is great interest in the proteins which control cdc2 dephosphorylation. Further knowledge of these proteins and their regulatory functions would be useful because it would provide a basis for a better understanding of cell division and, possibly, an approach to altering how it occurs.

Summary of the Invention

For the first time, a key aspect of control of MPF activation and, thus, entry into mitosis, has been demonstrated. That is, B-type cyclins have been shown to activate cdc25 PTPase and a cdc25 protein has been shown to be able to stimulate directly the kinase activity of pre-MPF, resulting in activation of the M-phase kinase. As a result, it is now possible to design approaches to regulating entry into mitosis and, thus, regulate the cell cycle.

As described herein, Applicant has isolated two previously undescribed human cdc25 genes, designated cdc25 A and cdc25 B, and has established that human cdc25 is a multigene family, consisting of at least three members. As further described herein, cdc25 A and cdc25 B have been shown to have an endogenous tyrosine phosphatase activity that can be specifically activated by B-type cyclin, in the absence of cdc2. It has also been shown for the first time that cdc25 phosphatases and B-type cyclins interact directly and that cyclin B is a multifunctional class of proteins which serve, in addition to their recognized role as regulatory subunits for M-phase cdc2, a previously unknown and surprising role as activators of the cdc25 phosphatase. In addition, Applicant has shown that, in Xenopus, cdc25 levels do not change, either during meiotic maturation or early embryonic division cycles; that cdc25 physically associates with a cdc2/cyclin B complex in a cell cycle dependent manner; that the maximal association between cdc25 and the cdc2/cyclin B complex occurs just before or at the time of maximal kinase activity (of cdc2); and that the cdc2 associated with cdc25 is tyrosine dephosphorylated and active as a kinase. In addition, as a result of the work described herein, it is now evident that in Xenopus, cyclin is the only protein that must be synthesized during each round of activation and inactivation of MPF. It had previously been proposed that cyclin must accumulate to a critical threshold before pre-MPF is activated. However, it is reasonable, based on the work described herein, to suggest that this threshold marks the point at which sufficient cyclin B has accumulated to allow activation of the continuously present cdc25 phosphatase (which, in turn, stimulates kinase activity of pre-MPF).

As also described herein, a surprising observation has been made as a result of comparison of the amino acid

sequences of newly discovered cdc25 A and cdc25 B gene products with known tyrosine protein phosphatases (PTPases) and other proteins involved in the cell cycle. That is, it has been shown that the region of cdc25 immediately C-terminal to the putative catalytic domain is not highly related to that of other known PTPases. Particularly interesting is the fact that this region within PTPases includes sequence similarity to cyclins, particularly B-type cyclins, and that cdc25 proteins have no equivalent "cyclin region". The newly found cyclin region is almost immediately adjacent to the domain implicated in the catalytic function of the PTPases and cdc25 protein. As a result of these findings, particularly the observation that cdc25 protein lacks a motif, shared by cyclin and other PTPases, that may be an activating domain, it is reasonable to suggest that in the case of cdc25, the activating domain is provided "in trans" by intermolecular interaction with cyclin.

As a result of the work described herein, new approaches to regulating the cell cycle in eukaryotic cells and, particularly, to regulating the activity of tyrosine specific phosphatases which play a key role in the cell cycle, are available. Applicant's invention relates to methods of regulating the cell cycle and, specifically, to regulating activation of cdc2-kinase, through alteration of the activity and/or levels of tyrosine phosphatases, particularly cdc25 phosphatase, and B-type cyclin, or through alteration of the interaction of components of MPF, particularly the association of cdc25 with cyclin, cdc2 or the cdc2/cyclin B complex. The present invention also relates to agents or compositions useful in the method of regulating (inhibiting or enhancing) the cell cycle. Such agents or compositions are, for example, inhibitors (such as low molecular weight peptides or compounds, either organic or inorganic) of the

catalytic activity of tyrosine specific PTPases (particularly cdc25), blocking agents which interfere with the interaction or binding of the tyrosine specific PTPase with cyclin or the cyclin/cdc2 complex, or agents which interfere directly with the catalytic activity of the PTPases.

Applicant's invention also relates to cdc25 A, cdc25 B and additional members of the cdc25 multigene family and to methods and reagents (e.g., nucleic acid probes, antibodies) useful for identifying other members of the cdc25 family, particularly those of mammalian (e.g., human) origin.

Applicant's invention also includes a method of identifying compounds or molecules which alter (enhance or inhibit) stimulation of kinase activity of pre-MPF and, thus, alter (enhance or inhibit) activation of MPF and entry into mitosis. The present method thus makes it possible to identify agents which can be administered to regulate the cell cycle; such agents are also the subject of this invention.

The present method makes use of a cell cycle-specific target and, thus, provides a highly specific mechanism-based screen for agents (compounds or molecules) which alter mitosis, particularly antimitotic agents. In the subject method, an agent is assessed for it's effect on the essential cell cycle-regulating component, cdc25 (e.g., cdc25A, cdc25B, cdc25C).

In particular, the agent to be assessed for its ability to inhibit cdc25 tyrosine phosphatase activity is combined with cdc25 and a substrate of cdc25 tyrosine phosphatase activity. The resulting combination is maintained under conditions appropriate for cdc25 to act upon the substrate. It is then determined whether cdc25 acted upon the substrate when the compound being assessed was present; the extent to which cdc25 acts upon the

substrate in the presence of the compound is compared with the extent to which cdc25 acts on the substrate in the absence of the compound (in comparison with a control). If cdc25 activity is less in the presence of the compound, the compound is an inhibitor of cdc25.

More particularly, a potential antimitotic agent (i.e., an agent to be assessed for an antimitotic effect) is combined with cdc25, which is either cdc25 protein or a fusion protein (e.g., recombinant $p80^{cdc25}$ present in a two-component fusion protein in which cdc25 is joined with a second component, such as glutathione-S-transferase). Subsequently, the effect of the potential antimitotic agent on the phosphatase activity of cdc25 is determined. $p80^{cdc25}$ protein has been shown, as described herein, to have p-nitrophenylphosphate phosphatase activity. Thus, the inhibitory effect of the agent being tested on cdc25 can be assessed using p-nitrophenylphosphate or inactive cyclin/cdc2 as substrate. Results obtained (e.g., the extent of inhibition of cdc25 phosphatase activity) are particularly valuable, since they demonstrate the effect of the agent tested on a target which is particularly well suited for detecting antimitotic agents because of its direct role in controlling entry of cells into M phase.

Brief Description of the Figures

Figures 1A-F are the nucleotide sequence of cdc25 A and the nucleotide sequence of cdc25 B. Panel A , sequence of cdc25 A cDNA (SEQ ID NO. 1). Panel B, sequence of cdc25 B (SEQ ID NO. 3). Below the nucleotide sequence is the translation in standard single letter amino acid code. In each sequence, the presumed initiating methionine is underlined. An in-frame stop codon upstream of the initiating AUG codon in the cdc25 A sequence is in bold and in each sequence, the terminating codon is marked by an asterisk.

Figure 2 shows the homology of cdc25 proteins. The amino acid sequences of cdc25 A and cdc25 B were aligned with human cdc25 C (formerly CDC25Hs), string (Stg) and S. pombe cdc25 (25Sp) using the FASTA program. Identical amino acids are boxed. In cases of only two alternative amino acids at a particular site a box is also used. Dashes within the sequences indicate individual amino acid gaps created by the computer to generate optimal alignment.

Figures 3A-B provide proof that human cdc25 A is essential for mitosis. Figure 3A is a graphic representation of the mitotic index of a population of the HeLa cells microinjected at time zero with the affinity-purified anti-cdc25A antibodies. Control cells were microinjected with the IgG fraction of the preimmune serum. Figure 3B is a graphic representation of the estimation of cell numbers in islands of HeLa cells injected at time zero with control or experimental anticdc25A affinity purified antibodies.

Figures 4A-C show activation of cdc25A phosphatase by mitotic cyclins. Human GST-cdc25 A fusion protein was used to assay release of 32p: substrates were tyrosine phosphorylated, reduced carboxamidomethylated, maleylated lyzosyme (RCML) (A); cdc2-derived peptide (B); or PNPP (C). A410 indicates adsorbance at 410 nm.

Figure 5 is a graphic representation of dose-dependent activation of the cdc25 A by cyclin B1. Bars indicate the standard error in three experiments.

Figure 6 shows inhibition of cdc25 phosphatase activity by p13 (Sucl). In the left panel, cdc25 A (10 pmoles) and right panel, cdc25 B (10 pmoles) was used. Bars indicate the standard error in three independent experiments.

Figures 7A-B show the alignment of the cdc25 proteins, PTPases and cyclins and a model of a proposed relationship

between PTPases and the M-phase kinase and cdc25 phosphatase. Panel A depicts the alignment, in which CA indicates the puative catalytic domain of the cdc25 and cytoplasmic tyrosine phosphatases, and CR indicates the cyclin related domain, present in tyrosine phosphatases but absent in cdc25 proteins. Panel B depicts a schematic representation of the hypothetical relationship between PTPases, and the M-phase kinase and cdc25 phosphatase.

Figure 8 is a graphic representation demonstrating that Xenopus cdc25 is required for activation of M-phase kinase. The ammonium sulfate fraction of the prophase oocyte extract was incubated in the presence of either PBS-2%BSA (filled diamonds) preimmune anti-cdc25 serum (oper circles; open diamonds), or purified anti-cdc25 antibody (filled rectangles; open rectangles). In two cases (open diamonds; open rectangles), soluble bacterially expressed yeast cdc25 protein (100 mg/ml) was added (indicated by arrows).

Figure 9 is a graphic representation evidencing periodic physical association of cdc25 and cdc2/cyclin B. Filled rectangles indicate histone H1 kinase activity of p13-Sepharose precipitates; open rectangles indicate amounts of cdc2 found in anti-cdc25 immunocomplexes by blotting with anti-cdc2 antibody.

Figure 10 is a schematic representation of the control by $p80^{cdc25}$ of activation of inactive pre-MPF (G2) to active MPF (M phase).

Figure 11 is evidence that the GST-cdc25a fusion protein dephosphorylates $p34^{cdc2}$ and activates the M phase-specific H1 kinase (MPF).

Figures 12A-B are graphic representation of GST-cdc25-pNPP phosphatase activity as a function of GST-cdc25A concentration (Figure 12A) and as a function of duration of assay (Figure 12B).

Figures 13A-B are graphic representation of GST-cdc25a activity as a function of DTT concentration (Figure 13A) and p-NPP concentration (Figure 13B).

Figure 14 is a graphic representation of the inhibitory effect of sodium orthovanadate on GST-cdc25A tyrosine phosphatase, in which phosphatase activity is expressed as % of activity in the absence of vanadate (mean ±SD).

Detailed Description of the Invention

The present invention relates to a method of regulating (inhibiting or enhancing) cell division and to agents or compositions useful for regulating the cell cycle. It further relates to two human genes, referred to as cdc25 A and cdc25 B, encoding tyrosine-specific phosphatases, the encoded tyrosine-specific phosphatases and additional members of the cdc25 multigene family, particularly additional human cdc25 genes, and their encoded products. In addition, the invention relates to a method of identifying agents which alter stimulation of kinase activity and thus alter entry of the cell into mitosis. The present invention also relates to an assay in which cdc25 tyrosine phosphatase, such as cdc25 protein or recombinant human cdc25 tyrosine phosphatase, is used as a cell cycle-specific target to screen for compounds which alter entry into mitosis (passage from late G2 into the M phase). Applicant's invention is based on identification of new cdc25 genes and the discovery that cdc25 proteins interact directly with and are specifically activated by B-type cyclins and activate cdc2 kinase.

Applicant has isolated two human cdc25 genes, designated cdc25 A and cdc25 B, and has thus established that human cdc25 is a multigene family of at least three members. The three human cdc25 proteins (cdc25 A, cdc25 B and the previously identified cdc25 protein) have been

shown to have approximately 40% identity in the most conserved C-terminal region. The cdc25 A and cdc25 B proteins can be classified as cdc25 proteins by a variety of independent criteria.

As shown herein, the cdc25 A gene product and cdc25 B gene product have endogenous tyrosine phosphatase activity in vitro which is stimulated several-fold, in the absence of cdc2, by cyclin B1 or cyclin B2. As is also shown herein, stable association occurs between cdc25 A and cyclin B1/cdc2 in human cells, specifically HeLa cells. These findings indicate that B-type cyclins are multi-functional proteins which not only are M-phase regulatory subunits, but also activate the cdc25 tyrosine phosphatase which, in turn, acts upon cdc2.

A region of amino acid similarity between cyclins and cytoplasmic tyrosine phosphatases has been identified and shown not to be present in cdc25 phosphatases, suggesting that the common motif represents an activating domain which must be provided to cdc25 by cdc25-cyclin B intramolecular interaction. Specifically, visual comparison of cdc25 A and cdc25 B with known tyrosine phosphatases (PTPases) and other proteins involved in cell cycle control resulted in the unexpected observation that a region of cdc25 immediately C-terminal to the putative cdc25 catalytic domain is not highly related to other known PTPases and that this newly found motif within the PTPases includes sequence similarity to cyclins, particularly of the B-type. Alignment of amino acid sequences of the cdc25 homologs and a diverse group of protein tyrosine phosphatases (PTPs) demonstrated that a C-terminal fragment of approximately 200 amino acid residues is a conserved protein motif which resembles the proposed catalytic center of viral and mammalian PTPases (see Example 1 and Figure 2).

Applicant has shown that the two new human cdc25 genes encode proteins functionally related to that encoded by the fission yeast cdc25 (Example 2). One of the human cdc25 genes (cdc25 A) has been shown to act in mitosis in human cells (Example 3), which arrest in a "rounded up" mitotic state after microinjection of anti-cdc25 A antibodies. Thus, Applicant has shown for the first time that the PTPase is necessary for cell division, Applicant has also shown that cell division is inhibited by anti-cdc25 A antibodies, which are, thus, a cytotoxic agent.

Surprisingly, it has also been shown that the endogenous phosphatase activity of cdc25 A and cdc25 B proteins purified from E. coli is directly activated by stoichiometric addition of B-type cyclin, in the absence of cdc2 (Examples 4 and 5), thus showing that B-type cyclins have a multifunctional role in this stage of cell division. This clearly demonstrates specificity between cyclins in their role as activators of cdc25. Until this finding, it has proved difficult to demonstrate differences in substrate specificity among members of the cdc2/cyclin family, although a variety of lines of evidence have suggested that cyclins of different classes have specific roles at particular stages of cell division. The cdc25 A protein has been shown to be present in a complex with both cyclin B1 and cdc2 (Example 5).

Applicant has also determined that Xenopus oocytes contain a relative of cdc25, designated p72, which can directly stimulate the M-phase kinase in vitro and is essential for activation of the M-phase kinase in cell-free lysates. As described herein, the abundance of p72 does not change in Xenopus embryos during the cell cycle. p72 has been shown to directly associate with cdc2/cyclin B in a cell cycle dependent manner, reaching a peak at M-phase. The M-phase kinase which associates with

p72 has been shown to be tyrosine dephosphorylated and catalytically active. As a result, it is reasonable to conclude that cdc25 triggers cdc2 activation by a mechanism which involves periodic physical association between cdc25 and the cyclin B/cdc2 complex, and that it is the association between cdc2/cyclin B and cdc25 which is required. It is also reasonable to conclude that mitotic control can be effected by mechanisms other than transcriptional regulation of the cdc25 gene.

As a result of Applicant's findings concerning the role of cdc25 in cell division, an assay is now available in which cdc25 is used as a cell-cycle specific target to screen for compounds which alter a cell's entry into the mitosis phase of cell growth. Results of the assay (i.e., the ability of the compound being tested to inhibit cdc25) are determined by known techniques, such as colormetrically, by immunoassay techniques or by detecting enzymatic activity (e.g., histone kinase activity).

The following describes Applicant's isolation and characterization of two new human cdc25 genes; demonstration of the multifunctional role of B-type cyclin in mitosis; the unexpected observation of a common amino acid sequence or motif present in PTPases and cyclins but absent in cdc25, and the determination that the motif resembles the proposed catalytic center of viral and mammalian PTPs; demonstration of a specific interaction between cdc25 phosphatases and B-type cyclins; and demonstration that the level of cdc25 in Xenopus oocytes does not change during the cell cycle. As a result of the work described, novel methods and compositions for cell cycle regulation are available, as well as an assay for compounds which alter cell cycle regulation. These methods, compositions, and assay are also described below.

Isolation and Characterization of Two New Human cdc25 Genes Which Are Members of a Multigene Family

Two new human cdc25 genes have been isolated, establishing the fact that in humans, cdc25 is a multigene family that consists of at least three members. The three human cdc25 proteins share approximately 40% identity in the most conserved C-terminal region. The two newly discovered cdc25 genes, cdc25 A and cdc25 B, can be classified as cdc25 proteins by a variety of quite independent criteria. First, they share sequence similarity with other members of the family. Second, cdc25 A and cdc25 B can each rescue a mutant cdc25-22 strain of fission yeast. Third, injection of antibodies prepared against a peptide comprising part of the cdc25 A protein into proliferating HeLa cells causes their arrest in mitosis. Fourth, cdc25 A protein eluted from immunocomplexes can activate the latent histone kinase activity of cdc2. Fifth, both cdc25 A and cdc25 B purified from E. coli display an endogenous tyrosine phosphatase activity.

The cdc25 Multigene Family

As described, it has now been shown that in humans, there are at least three cdc25 genes and possibly more. In fission yeast, only one essential cdc25 gene has been identified to date (Russell, P. and P. Nurse, Cell 45:145-153 (1986)). Likewise, a single essential mitotic B-type cyclin has been described in this yeast (Booher, R. and D. Beach, EMBO J. 7:2321-2327 (1988)). Two mitotic B-type cyclins have been found both in frog and humans (Minshull, J. et al., Cell 56:947-956 (1989)). Presumably, there is some differentiation of function between different members of the cdc25 and B-type cyclin families in vivo. Genetic studies in budding yeast, in which multiple B-type cyclins have been found, give some general

hint that this is the case (Surana, U. et al., Cell 65:145-161 (1991); Ghiara, J.B. et al., Cell 65:163-174 (1991)). However, both cyclin B1 and B2 could activate cdc25 A in vitro. One might postulate that different human cdc25 genes activate different cyclin B/cdc2 complexes in vivo and this may explain why injection of anti-cdc25 A serum into HeLa cells causes arrest in mid-mitosis, rather than in interphase.

It should be noted that regulation of cdc2 by tyrosine phosphorylation has currently only been described with respect to the cdc2/cyclin B enzyme. However, in certain contexts, it has been possible to substitute cyclin B with cyclin A (Swenson, K.I., et al., Cell 47:861-870 (1986)); Pines, J. and T. Hunt, EMBO J. 6:2987-2995 (1987)), and indeed human cyclin B2 was isolated by virtue of its ability to rescue a cn-deficient strain of budding yeast (Xiong, Y. et al., Cell 65:691-699 (1991)). In the work described herein, cyclin A could not activate cdc25 A or cdc25 B (not shown). This does not preclude, however, the existence of undiscovered cdc25-related phosphatases, that might be specifically activated by cyclin A. It is also presently unknown whether relatives of cdc2, such as cdk2 (formerly egl, Paris, J. et al., Proc. Natl. Acad. Sci. USA 88:1039-1043 (1991); Elledge, S.J. and M.R. Scottswood, EMBO J. 10:2653-2659 (1991)), that can bind cyclin A (Tsai, L-H. et al., Nature 353:174-177 (1991)), are subject to regulation by tyrosine phosphorylation and, hence, might require a cdc25 relative for activation.

Multifuncitonal Role Of B-type Cyclin In Mitosis

A particularly striking observation described herein is the demonstration that the endogenous phosphatase activity of cdc25 A and cdc25 B proteins purified from E. coli can be directly activated by stoichiometric addition

of B type cyclins. Specificity of this effect is shown by the inability of either cyclin A or cyclin D1 to display any such stimulation. A variety of lines of evidence suggest that cyclins of different classes have specific roles at particular stages of the division cycle (Booher, R. and D. Beach, EMBO J. 6:3441-3447 (1987); Booher, R. and D. Beach, EMBO J. 7:2321-2327 (1988); Nash, R. et al., EMBO J. 7:4335-4346 (1988); Hadwiger, J.A. et al., Proc. Natl. Acad. Sci. USA 86:6255-6259 (1989); Richardson, H.E. et al., Cell 59:1127-1133 (1989); Cross, F., Mol. Cell. Biol. 8:4675-4684 (1980); Wittenberg, C. et al., Cell 61:225-237 (1990); Draetta, G. et al., Cell 56:829-838 (1989); Giordano, A. et al., Cell 58:981-990 (1989); Pines, J. and T. Hunter, Nature 346:760-763 (1990); Xiong, Y. et al., Cell 65:691-699 (1991); Lew, D.J. et al., Cell 66:1-10 (1991); Koff, A. et al., Cell 88:1-20 (1991)). However, it has proved difficult to demonstrate differences in substrate specificity between members of the cdc2/cyclin family in vitro, and all known cyclins can rescue a CLN-deficient strain of budding yeast. The present experiments vividly demonstrate specificity between different cyclins in their role as activators of cdc25.

Certain evidence, both genetic and biochemical, suggests that cdc2 is a physiological substrate of cdc25 phosphatases (Gould, K. and P. Nurse, Nature 342:39-45 (1989); Kumagai, A. and W.G. Dunphy, Cell 64:903-914 (1991); Strausfeld, U. et al., Nature 351:242-245 (1991); Gautier, J. et al., Cell 67:197-211 (1991)). cdc2 was not used as a substrate in the present study because it binds to cyclins and, thus, potentially becomes altered as a phosphatase substrate; therefore, the issue of cdc25 substrate specificity has not been addressed directly. However, the finding of activation of cdc25, specifically by B-type cyclins, strengthens the conclusion that

cdc2/cyclin B is the relevant substrate _in vivo_. Demonstration of activation of cdc25 when artificial PTPase substrates were used leads to the conclusion that cyclins are able to interact with cdc25 in the total absence of cdc2 protein. _In vivo_, it is expected that this interaction occurs in the context of the cdc2/cyclin B pre-MPF complex. The above-described work demonstrates that B-type cyclins have at least two roles. First, they bind stoichiometrically with cdc2 to regulate the substrate specificity (Draetta, G. _et al_., _Nature_ _336_:738-744 (1989); Brizuela, L. _et al_., _Proc. Natl. Acad. Sci. USA 86_:4362-4366 (1989)) and the intracellular localization of the catalytic subunit (Booher, R.N. _et al_., _Cell 58_:485-497 (1989)). Second, they appear to have an independent function: the activation of cdc25 PTPase.

Genetic studies in fission yeast and Drosophila indicate that cdc25 is a dose-dependent activator of mitosis (Russell, P. and P. Nurse, _Cell 45_:145-153 (1986); Edgar, B.A. and P.H. O'Farrell, _Cell 57_:177-187 (1989)), whereas the cdc13 encoded B-type cyclin is essential for M-phase, but does not serve as a dose-dependent activator. Indeed, in many cell types, including the fission yeast, B-type cyclins accumulate and associate with cdc2 long before the tyrosine dephosphorylation event at the onset of M-phase (Booher, R.N. _et al_., _Cell 58_:485-497 (1989)). In some somatic cell types, the cdc25 gene is under transcriptional control, and very probably the cdc25 protein itself is regulated in a variety of ways that are not presently understood. In the early embryos of Xenopus, a somewhat different situation holds. As shown herein, the abundance of cdc25 is invariant during the cell cycle. Cyclin is the only protein that has to be synthesized during each round of activation and inactivation of MPF (Murray, W.W. _et al_., _Nature_ _339_:280-286 (1989)). It has been proposed that, in this context, cyclin must

accumulate to a critical threshold before pre-MPF is activated (Evans, T. et al., Cell 33:389-396 (1983); Pines, J. and T. Hunt, EMBO J. 6:2987-2995 (1987); Minshull, J. et al., Cell 56:947-956 (1989); Murray, A.W. and M.W. Kirshner, Nature 339:280-286 (1989)). Based on work described herein, it appears that this threshold marks the point at which sufficient cyclin has accumulated to allow activation of the continuously present cdc25 phosphatase.

The present findings may throw light on the long obscure phenomenon of MPF autoactivation. If a small amount of MPF is injected into a frog oocyte, a much larger amount can subsequently be retrieved (Masui, Y. and C.L. Markert, J. Exp. Zool. 171:129-146 (1971); Smith, L.D. and R.E. Ecker, Dev. Biol. 25:232-247 (1971)). The present work shows that in this situation, the abundance of cdc2, cyclin B and cdc25 do not change (Gautier, J. and J. Mailer, EMBO J. 10:177-182 (1991); see also Example 11). It has been implicitly assumed that active cdc2/cyclin B phosphorylates some protein (possibly cdc25 itself), causing the activation of cdc25 and, thus leading to further activation of pre-MPF. This may be correct, but if cyclin B directly activates cdc25 in the absence of cdc2, as shown herein, all of the elements needed for an autoactivation loop exist among the cdc2, cyclin B and cdc25 proteins themselves.

A Common Motif in PTPases and Cyclins

Alignment of the cdc25 proteins, PTPases and cyclins was performed, as shown in Figure 7A. Tyrosine phosphatases were aligned with each other as described in Guan, K. et al., (Nature 350:359-362 (1991)) and cdc25 proteins as described in Gautier, J. et al., (Cell 67:197-211 (1991)). The cyclin alignment was done by visual inspection. Only identity or similarity (V or I)

within at least three members of one gene family and a minimal of two members of other family is boxed. Visual comparison of cdc25 A and B with known tyrosine PTPases, and also other proteins involved in cell cycle control, resulted in the following unexpected observations. First, the region of cdc25 that is immediately C-terminal to the putative catalytic domain (CA) is not highly related to other known PTPases, such as cytoplasmic PTPases from higher eukaryotes and the vaccinia virus serine-tyrosine phosphatase (VH-I, Guan, et al., Nature 350:359-362 (1991); Figure 7A). Second and more interestingly, this region within the PTPases was found to contain sequence similarity to cyclins, particularly of the B-type (Figure 7A). The similarity was detected immediately at the junction of the so-called cyclin-box and included some nearly invariable residues among cyclins. The alignment in Figure 7A optimizes the similarities between cdc25 proteins and PTPases, and also between PTPases and cyclins, but ignores the much greater homology within each of the three groups of proteins. In the region of similarity between PTPases and cyclins, referred to as the cyclin region (CR), there is no equivalent in the cdc25 proteins.

The newly found motif lies almost immediately adjacent to the domain (V/IXHCXXXXR), that has been directly implicated in the catalytic function of the PTPases and cdc25 protein (Krueger, N.S. et al., EMBO J. 9:3241-3252 (1990); Guan, K. and J.E. Dixon, Science 249:553-556 (1990); Guan, K. et al., Anal.Biochemistry 192:262-267 (1991); Gautier, J. et al., Cell 67:197-211 (1991)). This finding allows the following speculation. The catalytic activity of the other PTPases is considerably greater than that of cdc25, at least as determined in this study. cdc25 lacks the motif that is shared by cyclins and other PTPases. This motif may be an acti-

vating domain which, in the case of cdc25, is provided in "trans" by intermolecular interaction with cyclin (Figure 7B), although in most PTPases it functions in "cis".

There is some similarity between PTPases and all of the classes of cyclin, whereas only B-type cyclins can activate cdc25. It is apparent, however, that the similarity is greatest between PTPases and cyclins of the B class. The differences between the various classes of cyclins within this region might be related to the specific ability of B but not A or D-type cyclins to activate cdc25 A.

Specific Interaction of cdc25 with Cyclin B

As shown in Example 13, cdc25 stably associates with a cdc2 complex and this interaction is periodic during the division cycle of Xenopus embryos. Human cyclin B1 is found in the complex with cdc25 A, as described in Example 5. It seems likely that the periodicity of the interaction between cdc25 and cdc2 is mediated at least in part by periodic accumulation and degradation of cyclin during the cell cycle.

As described herein, it has been established that cdc25 can function as an enzyme with respect to RCML, PNPP and cdc2 derived peptide substrates. A low observed catalytic rate was evident and may reflect the use of RCML or peptide as an artificial substrate. However, it is not clear what catalytic rate is required *in vivo*. If cdc25 does indeed associate with cdc2/cyclin B as suggested herein (Example 9 and Figure 7), the PTPase may not function in a conventional catalytic reaction, but rather only after formation of a cdc25/cyclin B/cdc2 complex. Under such conditions, the catalytic reaction is essentially intramolecular and Michaelis/Menten kinetics do not pertain.

Inhibition by p13 of Human cdc25 Phosphatase Activity

The p13 protein encoded by the sucl gene is an essential subunit of the cdc2 protein kinase. The gene was isolated by virtue of its ability to rescue a fission yeast cdc2-33 allele on a multicopy plasmid (Hayles, J. et al., EMBO J. 5:3373-3379 (1986)). However, overexpression of the gene is inhibitory for mitosis (Hindley, J. et al., Mol. Cell. Biol. 7:504-511 (1987); Hayles, J. et al., Mol Gen. Genet. 202:291-293 (1986)). In vitro, p13 can inhibit activation of pre-MPF (Dunphy, W. et al., Cell 54:423-431 (1988); Dunphy, W. and J.W. Newport, Cell 58:181-431 (1989)).

The present work may clarify two previously confusing issues related to these observations. First, p13 can bind to cdc2 in the absence of cyclins (Brizuela, L. et al., EMBO J. 6:3507-3514 (1987); see also Example 6), but activation of cdc2/cyclin B that is pre-bound to p13-sepharose can be inhibited by excess exogenous p13 (Jessus, C. et al., FEBS LETTERS 266:4-8 (1990)). By contrast, fully activated cyclin B/cdc2 is not inhibited by excess p13 (Dunphy, W. et al., Cell 54:423-431 (1988); Arion, D. et al., Cell 55:371-378 (1988); Maijer, L. et al., EMBO J. 8:2275-2282 (1989)). This suggests, as previously proposed (Jessus, C. et al., FEBS LETTERS 266:4-8 (1990)), that there are at least two binding sites for p13. One is presumably a high affinity binding site on cdc2 itself, that accounts for the extraordinary efficiency of p13-sepharose chromatography. The other site, of lower affinity requiring p13 in the 20 micromolar range, does not affect fully activated cdc2/cyclin B, but can inhibit activation of pre-MPF. Because direct inhibition of cdc25 A endogenous phosphatase activity by p13, in the total absence of cdc2, has been observed (Example 6), it is reasonable to attribute the second binding site not to cdc2, but to cdc25. This is probably

an unstable interaction, quite unlike that between p13 and cdc2. A schematic representation of the hypothetical relationship between PTPases, the M-phase kinase and cdc25 phosphatase, is shown in Figure 7B. The association between cdc2 and p13, and between cyclin and cdc2, is well documented. The interaction of cdc25 and cyclin is also proposed here, p13 is proposed to have a low affinity interaction with cdc25. CA is the catalytic domain of PTPases and CR is a region of similarity between PTPases and cyclins.

Second, there has been some dispute concerning the inhibition of cdc25 by p13 in different experimental contexts. In some cases, p13 has been inhibitory (Gautier, J. et al., Cell 67:197-211 (1991)) and in other cases, it has not (Kumagai, A. and W.G. Dunphy, Cell 64:903-914 (1991)). As described herein under the conditions used, cdc25 A is inhibited by p13, and cdc25 B is not. The two proteins have many regions of structural dissimilarity that could readily account for this effect.

cdc25 Does Not Change in Abundance During the Cell Cycle

Surprisingly, the Xenopus cdc25 does not oscillate in abundance, either during meiotic maturation, or during the early embryonic division cycles. The protein does, however, physically associate with the cdc2/cyclin B complex in a cell cycle dependent manner (see Examples 5 and 10). Maximal association is found just before or at the time of maximal kinase activity (see Examples 11 and 13, and Figure 9). The cdc2 that is associated with cdc25 is tyrosine dephosphorylated and active as a histone H2 kinase. The association between cdc25 and the cdc2/cyclin B complex could be mediated either by cdc2 or by cyclin B. As described herein, B-type cyclins were shown to be able to directly activate the intrinsic PTPase activity of cdc25 proteins in the absence of cdc2. This suggests that

the interaction between cdc25 and the cdc2/cyclin B complex is probably mediated by cyclin.

These results bear upon the mechanism by which cdc2 becomes activated at M-phase. cdc25 acts in mitosis to cause the tyrosine dephosphorylation of cdc2, as described herein. The demonstration of direct physical association between cdc25 and the cdc2/cyclin B complex is entirely consistent with this hypothesis. The finding that approximately 5% of cdc2 associates with cdc25 at M-phase raises certain questions. It is possible that one molecule of cdc25 binds to cdc2/cyclin B, activates the kinase and then dissociates to repeat the process in a conventional catalytic mechanism. Alternatively, a single molecule of cdc25 might activate only a single molecule of pre-MPF in a stoichiometric mechanism. Only a fraction of the total amount of cdc2 (10% of the cellular cdc2 content, as described in Kobayashi A.H. <u>et al</u>., <u>J. Cell Biol. 114</u>:755-765 (1991)) is bound to cyclin B and activated at M-phase in Xenopus eggs. The finding that only 5% of total cdc2 is associated with cdc25 at mitosis might reflect the relatively low abundance of cyclin B compared to cdc2, if the interaction is mediated by cyclin B. This is confirmed by the fact that, in comparison to the 5% cdc25-associated cdc2, a larger amount of cyclin B2 is found in association with cdc25 (17% of the full cellular amount of cyclin B2). Moreover, a considerable fraction of cdc25 is involved in this association (20% of the cellular content).

<u>Identification of Additional cdc25 Genes and Cell Cycle Regulation by the Present Invention</u>

Using methods described herein, such as in Examples 1 and 7, additional members of the human cdc25 gene family and cdc25 genes in other organisms can be identified and isolated; the encoded products can be identified as well.

For example, all or a portion of the nucleotide sequence of the cdc25 A gene or the cdc25 B gene (see Figure 1) can be used in hybridization methods or amplification methods known to those of skill in the art (Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, NY (1989)). For example, a nucleotide sequence which is all or a portion of the cdc25 A gene or the cdc25 B gene can be used to screen a DNA library of human or nonhuman origin for additional cdc25 genes. DNA sequences identified in this manner can be expressed and their products analyzed for tyrosine specific phosphatase activity, such as by the methods described herein (see Experimental Procedures and Example 2). Hybridization conditions can be varied as desired. If a nucleotide sequence which is exactly complementary to the probe used is to be isolated, conditions of either high or low stringency can be used; if a nucleic acid sequence less related to those of the probe is to be identified, conditions of lower stringency are used. The present invention includes the cdc25 A and cdc25 B genes and equivalent cdc genes; equivalent genes, as used herein, are nucleic acid sequences which hybridize to all or a portion of the cdc25 A or cdc25 B gene or a complement of either gene, and encode a tyrosine PTPase which has substantially the same catalytic function as the cdc25 A or cdc25 B gene product. The polymerase chain reaction and appropriately designed primers can also be used to identify other cdc25 genes. Alternatively, an anti-cdc25 A or anti-cdc25 B antibody can be used to detect other (recombinant) cdc25 gene products expressed in appropriate host cells transformed with a vector or DNA construct thought to encode a cdc25 product. The cdc25 A gene, cdc25 B gene and equivalent cdc genes which are the subject of the present invention include those obtained from naturally occurring sources and those produced by

genetic engineering (cloning) methods or by synthetic methods. These genes can be used to produce the encoded cdc25 A, cdc25 B or other cdc25 gene product, which can, in turn, be used to produce antibodies specific for the product or to regulate cell cycle activation (cdc2 kinase activation), as described below.

The present invention also includes PTPase genes which encode PTPases which are related to cdc25 PTPases but are specifically activated by a non-B type cyclin (e.g., by cyclin A, cyclin D). These PTPases are referred to herein as cdc25-related PTPases and their activation by a cyclin, their ability to activate cdc2 or another molecule and their role in regulation of the cell cycle can be assessed using the methods described for determining the role of cdc25.

The present invention also provides a method by which the level of expression or activity of cdc25 PTPases in a cell can be determined and assessed (i.e., to determine if they increased, decreased or remained within normal limits). Because the cdc25 gene is increased (overexpressed) in certain tumor types, the present invention also provides a method of diagnosing or detecting overexpression related to those tumor cell types. In the method, a gene probe to detect and quantify the cdc25 gene in cells, or antibodies specific for the cdc25 PTPase can be used.

<u>Assay for Compounds Which Alter cdc25 Function/Entry into Mitosis</u>

A method of inhibiting activation of cdc25 PTPases, activation of cdc2 kinase(s) and, thus, initiation of mitosis (cell division) is also possible. For example, activation of cdc25 PTPase is inhibited (reduced or prevented) by introducing into cells a drug or other agent which can block, directly or indirectly, complexing of

cdc25 with cyclin B or the cyclin B/cdc2 complex and, thus, directly block activation of the cdc25 and indirectly block activation of the cdc2 kinase. In one embodiment, complex formation is prevented in an indirect manner, such as by preventing transcription and/or translation of the cdc25 DNA and/or RNA. This can be carried out by introducing into cells antisense oligonucleotides which hybridize to the cdc25-encoding nucleic acid sequences, and thus prevent their further processing. It is also possible to inhibit expression of the cdc25 product by interfering with an essential cdc25 transcription factor. Alternatively, complex formation can be prevented by degrading the cdc25 gene product(s), such as by introducing a protease or substance which enhances their breakdown into cells. In either case, the effect is indirect in that a reduced quantity of cdc25 is available than would otherwise be the case. In another embodiment, activation of cdc25 PTPase is inhibited by interfering with the newly identified region of cyclin which has been shown to share sequence similarity with a region present in other PTPases, but not present in cdc25, and which appears to be provided to cdc25 in trans by intermolecular interaction with cyclin.

In another embodiment, activation of cdc25 PTPase is inhibited in a more direct manner by, for example, introducing into cells a drug or other agent which binds the PTPase and prevents complex formation with cyclin (and, thus, prevents PTPase activation). Alternatively, a drug or other agent which interferes in another manner with the physical association between cyclin and the PTPase (e.g., by intercalation), or which disrupts the catalytic activity of the enzyme can be introduced into cells. This can be effected, for example, by use of antibodies which bind the PTPase or the cyclin, or by a peptide or low molecular weight organic or inorganic

compound which, like the endogenous type B cyclin binds the cdc25 PTPase, but, unlike type B cyclin does not result in activation of the enzyme or results in its being disabled or degraded. Peptides and small organic compounds to be used for this purpose can be based on analysis of the amino acid sequences of B type cyclins or of the amino acid sequences of the cdc PTPase(s) involved. They can be designed, for example, to include residues necessary for binding and to exclude residues whose presence results in activation. This can be done, for example, by systematically mapping the binding site(s) and designing molecules which recognize or otherwise associate with the site(s) necessary for activation, but do not cause activation. One site of particular interest for this purpose is the region which, as described above, is missing in cdc25 PTPases and appears to be provided in trans by intermolecular binding of the cdc25 product and type B cyclin. At least three possible approaches are possible in this instance. First, a molecule (e.g., a peptide which mimics the binding site on type B cyclin for cdc25) can be introduced into cells; the molecule then binds cdc25 and blocks its interaction with cyclin. Second, a molecule mimicing the region of cdc25 which binds the type B cyclin molecule can be introduced into cells; the molecule then binds cyclin and blocks the cdc25-cyclin complex formation. Third, a molecule which inhibits or inactivates the putative activating domain on type B cyclin can be introduced into cells, thus preventing activation of the cdc PTPase.

In another embodiment, inhibitors of the catalytic activity of cdc25 PTPase are introduced into cells. Such inhibitors are low molecular weight agents, such as peptides and inorganic or organic compounds.

The present invention also includes a method of screening compounds or molecules for their ability to

inhibit the function of cdc25 protein or the binding of the cdc25 protein with the cyclin/cdc2 complex. For example, cells as described herein, in which a cdc25 gene is expressed, can be used. A compound or molecule to be assessed for its ability to inhibit cdc25 protein function or binding to the cyclin/cdc2 complex is contacted with the cells, under conditions appropriate for entry of the compound or molecule into the cells. Inhibition of the cdc25 protein or of complex formation will result in arrest of the cells or a reduced rate of cell division. Comparison with cell division of an appropriate control (e.g., the same type of cells without added test drug) will demonstrate the ability or inability of the compound or molecule to inhibit the cyclin. Alternatively, an in vitro assay can be used to test for compounds or molecules able to inhibit cdc25 PTPases or their binding to the cyclin/cdc25 complex. In this in vitro assay, the three components (cdc25 PTPase, cyclin and cdc2 (the latter two either individually or as a cyclin/cdc2 complex such as inactive cyclin/cdc2 complex from interphase cells) are combined with a potential cdc25 inhibitor. The activity of the potential inhibitor is assessed by determining whether cdc25 binds cyclin or cyclin/cdc2 complex or whether cdc2 is activated, as evidenced by histone kinase activity. This method can make use of the teachings of Jessus et al. (FEBS Letters 66:4-8 (1990)) and DuCommun and Beach (Anal. Biochem. 187: 94-97 (1990)), the teachings of which are incorporated herein by reference. For example, in an assay for cdc25 inhibitors, inactive cyclin/cdc2 complex can be placed in the wells, cdc25 and a test compound or molecule added to wells and cdc2 activation assessed. In the presence of a cdc25 inhibitor, cdc2 activation will be prevented or reduced (less than would occur in the absence of the test compound or molecule).

Existing compounds or molecules (e.g., those present in fermentation broth or a chemical "library") or those developed to inhibit the cyclin activation of its protein kinase can be screened for their effectiveness using this method. Drugs which inhibit cdc25 protein catalytic activity, inhibit complex formation or degrade or otherwise inactivate cdc25 are also the subject of this invention.

The present invention also includes an assay in which cdc25 tyrosine phosphatase, such as cdc25 protein or recombinant human cdc25 tyrosine phosphatase, is used to screen for compounds which alter entry into mitosis (passage from late G2 into the M phase of the cell cycle). In one embodiment of the assay, a colorimetric assay can be used to determine the ability of compounds to inhibit the cdc25 tyrosine phosphatase, which is an activator of the protein kinase MPF. As described herein, a glutathione-S-transferase/cdc25A tyrosine phosphatase fusion protein produced in *Escherichia coli* and purified displays a phosphatase activity towards p-nitrophenylphosphate. This fusion protein, designated GST-cdc25A, has been used to assess the inhibitory effect of compounds on cdc25 phosphatase activity. In a similar manner, as also described herein, other fusion proteins can be produced and used in the same or a similar assay format. These fusion proteins can differ from GST-cdc25A in either or both of their components. For example, a component other than GST (e.g., maltase binding protein) can be included in the fusion protein with cdc25A. Alternatively, another member of the cdc25 family (e.g., cdc25B, cdc25C) can be the tyrosine phosphatase component. In another embodiment, cdc25 protein is used.

The present method is a simple and rapid screening test which, in one embodiment, uses a fusion protein such as recombinant $p80^{cdc25}$, assayed through its

p-nitrophenylphosphate phosphatase activity, as a target to test for potential antimitotic compounds. The method has been carried out as a rapid calorimetric microtitration plate assay to test compounds currently used in cancer therapy, and a compound recognized to be a tyrosine phosphatase inhibitor. The therapeutic compounds tested did not display an ability to inhibit cdc25, in the assay as described; the reported tyrosine phosphatase inhibitor (vanadate) was shown, however, to totally inhibit cdc25. Thus, the present method has been shown to be useful in identifying compounds which inhibit an essential cell cycle-regulating component; it provides a highly specific screen for antimitotic drugs.

In one embodiment of the present method, a fusion protein which includes cdc25 is combined, under appropriate conditions, with: 1) an agent to be assessed for its effects on cdc25 and, thus, on passage from late G2 into the M phase; and 2) an appropriate cdc25 substrate, such as p-nitrophenylphosphate or inactive cdc2/cyclin B. The resulting combination is maintained for sufficient time for cdc25 to act upon the cdc25 substrate and the reaction is terminated (e.g., by gross alteration of the pH of the combination). Phosphatase activity of the combination is determined using a known technique, such as by measuring the optical density of the combination and comparing it with a predetermined standard or a control (e.g., a predetermined relationship between optical density and extent of cdc25 inhibition or a combination which includes the same components as the "test" combination except for the agent being assessed).

The fusion protein used in the present method can be produced by known genetic engineering techniques, as described in Example 14. That is, a DNA or RNA construct encoding the fusion protein is introduced into an appropriate host cell, in which the construct is

expressed, thus producing the fusion protein. The fusion protein is separated (and, preferably, purified) from the host cell and used in the assay. Alternatively, the fusion protein can be produced by joining the two separately produced components. As described in Example 15, a fusion protein in which the two components are glutathione-S-transferase and human cdc25A has been produced and used in the subject method.

In a second embodiment, cdc25 protein, such as cdc25A, cdc25B or cdc25C protein, can be used in the subject method. In this embodiment, cyclin/cdc2 can be used as the cdc25 substrate; an agent to be tested is combined with cdc25 protein and cyclin/cdc2 and the tyrosine phosphatase activity of cdc25 is assessed, as described above. Results are compared with a predetermined standard or with a control (see Example 14).

The cdc25 substrate used can be any synthetic or naturally-occurring substance toward which cdc25 demonstrates phosphatase activity. In the embodiment described herein, the cdc25A substrate used is p-nitrophenylphosphate. Other substrates which can be used include peptides that mimic the site of cdc2 phosphorylation or the full inactive cdc2/cyclinB pre-enzyme complex. Others can be identified by using known methods of determining phosphatase activity.

Agents to be tested for their ability to alter cdc25 tyrosine phosphatase activity can be those produced by bacteria, yeast or other organisms, or those produced chemically. The compounds tested herein, as described in Exmaple 18, included 15 drugs currently used in cancer therapy and vanadate, a recognized tyrosine phosphatase inhibitor. The 15 therapeutic agents showed no inhibitory activity. In contrast, vanadate was shown to totally inhibit GST-cdc25A phosphatase. The present method is useful to identify agents potentially effective as

antiproliferative agents and agents which are useful in treating or preventing inflammation or psoriasis, or other diseases relating to cell proliferation.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

EXPERIMENTAL PROCEDURES

The following experimental procedures were used in carrying out the work described in Examples 1-6.

Three highly degenerate primers corresponding to the consensus cdc25 protein sequence were designed taking into account homology between the *S. pombe* cdc25, Drosophila *string* and *S. cerevisiae* mihl gene products. 5' degenerate primers corresponding to the amino acid sequence IIDCRT/FP (or E) Y E (SIC-1: ATIATIGATTGCCGITA/TCCCITAC/TGA and SIC-2: ATIATIGATTGCCGITA/TCGAITAC/TGA) (SEQ ID NO. 5) and a 3' primer corresponding to the amino acid sequence I/V F H C E F (ST-C: A/TA/GAAC/TTCA/GCAA/GTGA/GAAA/G/TA), (SEQ ID NO. 6) where I corresponds to inosine, were prepared. The 50 ml PCR reaction mixture contained 50 mM KCl; 10 mM TrisHCl(pH 8.3); 1.5 mM $MgCl_2$; 0.01% gelatin; 0.2 mM each of dATP, dCTP, dGTP and dTTP; 0.5 unit of *Thermus aquaticus* (AmpliTaq DNA polymerase (Perkin-Elmer/Cetus)), 2 mM each of the 5' primers (SIC-I and SIC-2)) 5 mM of the 3' primer (ST-C) and 100 mg of human N-Tera cells cDNA library made in ggt10 by Jacek Skowronski (Cold Spring Harbor Laboratory). Four cycles of 94°C for 1 min, 40°C for 3 min and 72°C for 1 min were performed in a DNA thermal cycler (Perkin-Elmer/Cetus). The reaction products were separated on the 2% agarose gel and the expected size (approximately 160 bp) fragments were subcloned into Sma1-digested pBluescript SK(-) vector (Stratagene, La Jolla, CA). Nine clones were sequenced, with the sequence clearly indicating cloning of cdc25

homologues. Two different PCR products were detected: one of them was almost identical to recently cloned human cdc25 homologue (CDC25Hs, Sadhu, K. et al., Proc. Natl. Acad.Sci.USA 87:5139-5143 (1990)), and the other corresponded to a previously uncharacterized cDNA, here called cdc25 A. The N-Tera cdc25 A PCR-derived clone (p5wl) was used to screen the human N-Tera cell library at low stringency. After plaque purification, inserts of nine positive clones were subcloned into the EcoRI site of the pBluescript SK(-) plasmid. Inserts from two phages containing the entire open reading frame of the cdc25 A cDNA were analyzed by restriction mapping (plasmids 4g1.3 and 211.1, containing inserts of 2.4 and 3.9 kb). Plasmid 4g1.3 contained a deletion of 1.4 kb at the 3' untranslated region of the cDNA and was chosen for complete sequencing. Sequence analysis was performed on both strands using a chain termination method on an automated sequencing system (Applied Biosystems 373A).

Further analysis indicated that one of the original nine phage clones corresponded to a different cdc25 homolog; this is designated cdc25 B. This phage gave rise to two EcoRI fragments (0.9 and 1.5 kb) but did not represent a whole open reading frame. In order to obtain a complete cDNA, the same library was screened with the 0.9 kb EcoRI fragment and an insert representing a complete cDNA (3.0 kb) was subcloned via partial digestion with EcoRI into the pBluescript SK(-) vector. This was used for sequencing.

Production of Antipeptide Antiserum to Human cdc25 A and CDC25Hs

Peptides corresponding to the amino acid sequence CQGALNLYSQEELF-NH_2 (#143)(CDC25Hs or cdc25 C) and CKGAVNLHMEEEVE-NH_2 (#144)(cdc25 A) were synthesized at the Cold Spring Harbor Laboratory protein core facility,

HPLC-purified and coupled to keyhole limpet hemocyanine (KLH) and bovine serum albumin essentially as described (Draetta, G. et al., Nature 336:738-744 (1988)). Rabbits were injected with 200 mg of KLH-peptide conjugate every three weeks. Positive sera were obtained after three booster injections. Antibody (K143 and K144) were affinity purified on the BSA-peptide conjugates coupled to the CNBr-Sepharose (Pharmacia, Sweden) according to the manufacturer's instructions. No crossreactivity between peptide #134 and K144 antiserum with the other peptide was detected.

Rescue of the Fission Yeast cdc25 Temperature Sensitive Mutant

A 2.0 kb NcoI-BamHI fragment encoding amino acids 1-526 of human cdc25 A from the p4gl.3 plasmid were subcloned into NcoI-BamHI digested pARTN, resulting in the pARTN-cdc25 A construct harboring human cdc25 A cDNA in sense orientation to the constitutive adh promoter. pARTN is derived from the pART3 (McLeod, et al., 1987) by ligation of an NcoI linker (New England Biolabs) into the Smal site. An 2.4 kb Smal fragment from the p4xl.2 plasmid encoding amino acids 32-566 was subcloned into Smal digested pART3 vector (containing LEU2 marker) resulting in pARTN-cdc25 B cDNA. Both plasmids were transformed into *S. pombe* h+cdc25-22 leul-32 (SP 532) strain. Leu+ transformants were obtained at 26°C.

Cell Culture, Immunoprecipitation

HeLa cells (obtained from the ATCC) were grown at 37°C in Dulbecco modified Eagle's media (DMDM) supplemented with 10% fetal calf serum. For labelling, cells were washed with methionine minus media (Gibco) and supplemented with 1mci/ml ^{35}S-methionine (Translabel, ICN) for 6-8 hours. Cells were lysed essentially as described

(Draetta, G. et al., Nature 336:738-744 (1988)) or in the EB buffer (80 mM glycerophosphate, 15 mM $MgCl_2$, 20 mM EGTA, 1 mM DTT), supplemented with protease inhibitors (0.5 mM PMSF, 1 mg/ml of aprotinin, pepstatin, chymostatin, leupeptine, 30 mg/ml of TPCK, 15 mg/ml benzimidine). Lysates were precleared with protein A-Sepharose beads (Pharmacia) (20 ml of the 1:1 slurry); anti-human cdc25 A antiserum (K144) were added (1-5 ml); and after 8-10 hours immune complexes were precipitated with protein A-beads (20 ml of the 1:1 slurry). Beads were washed four times with the lysis buffer and resuspended in 20 ml 2x sample buffer (Laemmli, U.K. Nature 227:680-685 (1970)). Immunoprecipitated proteins were resolved on the 10% polyacrylamide gels containing SDS, and visualized by the autoradiography of the dried gel slabs (Anderson, S.J. et al., J. Virol. 51:730-741 (1984)). p13 beads were prepared and used to precipitate $p34^{cdc2}$ from HeLd as described earlier (Brizuela, L. et al., EMBO J. 6:3507-3514 (1987)).

Bacterial Expression of the cdc25 A and cdc B Phosphatase Assay

A plasmid containing the entire open reading frame of human cdc25 A was digested with Ncol (at amino acid 1), blunt ended with T4 DNA polymerase, heat inactivated, extracted with phenolchlorophorm, ethanol precipitated and digested with EcoRI. The resultant 2.0 kb fragment was gel-purified and ligated into pGEX-2T Smal/EcoRI digested vector. Resultant plasmid upon transformation into bacteria gave rise to a 90 kd IPTG-inducible protein. Expressed fusion protein was recovered as described (Smith, D.B. and K.S. Johnson, Gene 67:31-40 (1988)) on glutathione-Sepharose beads (Pharmacia), and eluted with 5 mM freshly prepared glutathione in 50 mM TrisHCl, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, at pH 8.0. For expression of

cdc25 B, plasmid p4xl.2 was cut with XbaI, then with SmaI (partially) and the 2.4 kb fragment was subcloned into SmaI/XbaI cut pGEX-KG vector (Guan, K. and J.E. Dixon, Science 249:553-556 (1991)). Expression of this construct resulted in IPTG-dependent synthesis of the 88 kD GST-cdc25 B fusion protein. Phosphatase activity of the purified cdc25 A protein (4.5 mg or 50 pmoles) was assayed in 0.5 ml 20 mM Tris HCl, pH 8.0, 1 mM EDTA, 0.1% b-mercaptoethanol, 20 mM p-nitrophenylphosphate (PNPP). Absorbance at 410 nm was determined using a molar absorptivity of $1.78 \times 10^4\ M^{-1}\ cm^{-1}$ to calculate the concentration of the p-nitrophenolate ion generated in the assay. For cdc25 B the assay was performed in the same buffer except at pH 8.8.

Reduced carboxamidomethylated and maleylated lysozyme (RCML) was obtained from N. Tonks in a ^{32}p-tyrosine phosphorylated form. Approximately 50% of the protein was phosphorylated. ^{32}P-labeled RCML was used in the phosphatase assay in 50 mM Tris HCl, pH 8.0, 50 mM NaCl, 0.1 mm EDTA, 1 mM DTT at a final phosphate concentration of 10-30 mM. Reactions (30-50 ml) were performed at 30°C for 10 or 20 min, and after addition of the fatty acid free bovine serum albumin (BSA, Sigma) to 2 mg/ml, proteins were precipitated with 200 ml of 20% trichloro-acetic acid, vortexed, incubated at -70°C for 5 min, thawed, spun in an Eppendorf centrifuge for 5-10 min at the maximal speed and 200 ml supernatants were counted in 2 ml Aquasol (NEN) for 10 min.

Peptide, corresponding to region of $p34^{cdc2}$ undergoing inhibitory tyrosine phosphorylation (NH2-CKKKVEKIGEGTYGVVYK) (SEQ ID NO. 7) (the peptide sequence which is additional to cdc2 and added to couple the peptide to the beads and/or proteins is underlined) was phosphorylated in vitro using bacterially produced v-Abl (Oncogene Sciences) at conditions described by the

manufacturer and purified on the Seppak column (Millipore). Final activity incorporated into peptide was 0.7×10^5 cpm/mg. Phosphatase activity of the cdc25 A protein against peptide (1 mg of peptide were used in each sample) was assayed at the same conditions as for RCML. Reaction mixture was incubated with acid charcoal as described (Streuli, M. et al., Natl. Acad. Sci. USA 86:8698-8702 (1989)) and 200 ml from total supernatant of 700 ml were counted as described above.

Expression of Cyclin Proteins

In order to express human cyclins in bacteria modified pGEX-3X vector (pGEX-Nco) was prepared by digesting it with SmaI, followed by ligation of the NcoI linker (described earlier in Experimental procedures); this resulted in a vector where cloning into NcoI site allowed the proper expression of the foreign cDNA. Human cyclin BI and A were synthesized by PCR and their sequence were fully confirmed. cyclin B1 cDNA in the pBluescript SK(-) was cut with NcoI/SmaI and the resultant 1.3 kb fragment was ligated into pGEX-Nco, digested with EcoRI, filled in with Klenow fragment and cut with NcoI. The sequence of cyclin A, including the first ATG codon, was changed to an ncoI site by PCR. To express cyclin A, plasmids containing the complete open reading frame for cyclin A (p4fl.1) were digested with NcoI and EcoRI and the resultant 1.4 kb insert was subcloned into pGEX-Nco cut with NcoI/EcoRI. Human cDNA encoding human cyclin B2 was obtained from Y.Xiong (unpublished), with the first ATG codon changed by PCR to NcoI site, this cDNA was digested with BamHI, blunt ended with T4 DNA polymerase, and digested with the NcoI, and the resultant 1.3 kb fragment was ligated in the pGEX3X-Nco vector prepared as described above for the ligation of cyclinB1 cDNA. Mouse CYL1 (cyclin D1) cDNA in the pGEX-3X vector was generous

gift from Dr. C. Sherr. Purification of the expressed cyclins was performed essentially as described (Smith, D.B. and K.S. Johnson, Gene 67:31-40 (1988); Solomon, M.J. et al., Cell 63:1013-1024 (1991)), except that after the first extraction, the cell pellets were resuspended in the 50 mM TrisHCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% glycerol, 2M urea and extracted for 10 min on ice. After centrifugation for 30-60 min at 15000 rpm on the RC-5B centrifuge (Beckman), the supernatant was filtered through 0.22 mm filter (Millipore) and applied on the 2 ml glutathione-Sepharose column (Pharmacia), equilibrated with the extraction buffer. columns were washed subsequently with the extraction buffer (10 ml), then with the same buffer lacking urea (10 ml), and fusion proteins were eluted in the same buffer supplemented with 10 mM glutathione. Eluted proteins were dialized into phosphatase assay buffer and concentrated by repeated dilution-concentration on the Amicon microconcentrators. Protease inhibitors (PMSF and benzimidine) were added to 0.5 and 5 mM subsequently, and the proteins were stored at 4°C for 2-3 days or used immediately on the same day. The Bradford assay was used to determine protein concentration.

Microinjection of Antibodies

For microinjection experiments HeLa cells were grown to 20-30 cells in an "island" and injected at time 0 with affinity purified K144 (1 mg/ml) further depleted on the #143 peptide conjugated BSA sepharose. The injection was done in buffer F (20 mM Tris HCl, pH 7.6, 20 mM NaCl, 50 mM KCl, 0.5 mM b-mercaptoethanol, 0.1 mM ATP). All cells in the particular "island" were microinjected and photographs were taken at 8, 18, 24 and 36 hours after microinjection. In a separate set of experiments cells were photographed at 8, 12, 18 and 24 hours after injection.

Microinjection of the protein A-Sepharose purified rabbit IgG from the preimmune serum served as a control.

Protein Kinase Assays

For protein kinase assays, p13 beads with bound $p34^{cdc2}$ kinase isolated from the HeLa cells (incubated in the presence of hydroxyurea (10 mM) for 22 hours followed by 4 hour release) were washed twice in the buffer containing 50 mM Tris HCl, pH 8.0, 1 mM EDTA, 1 mM DTT and incubated for 5 min at 30°C with the additives. Additives included buffer alone, or material eluted with the 0.1 M glycine/HCl, pH 2.5 from the cdc25 A immunoprecipitates, done in the presence or absence of 1 mg of an antigenic peptide (before addition material was neutralized with 1 M Tris HCl, pH 8.0). The precipitates were washed twice with 50 mM Tris HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT (PK-buffer), and finally resuspended in 2 volumes of PK buffer supplemented with 5 mM ATP, 10 mCi of $[\alpha\text{-}^{32}P]$ ATP (3000 Ci/mmol), and 50 mg/ml of histone H1. After incubation for 15 min at 30°C the reaction was stopped by polyacrylamide gel sample buffer containing SDS. Labeled proteins were separated on 10% polyacrulamide gels and detected by autoradiography.

EXAMPLE 1 ISOLATION OF cdc25 A AND cdc B cDNA

A human cdc25 genes has previously been described (Sadhu, K. et al. Proc. Natl Acad USA, 87:5139-5143 (1990)). Further members of what is now shown to be the human cdc25 family have been isolated by means of a PCR-based strategy. This strategy made use of three degenerate oligonucleotide primers designed to correspond to amino-acid regions of consensus between Drosophila melanogaster string (Edgar, B.A. and P.H. O'Farrell, Cell 57:177-187 (1989)), S. pombe cdc25

(Russell, P. and P. Nurse, Cell 45:145-153 (1986)) and S. cerevisiae mihl (Russell, P. et al., Cell 57:295-303 (1989)). Amplification of cDNA from a human N-Tera teratocarcinoma library, followed by cloning of the PCR products into a phagemid vector, allowed nucleotide sequencing of the fragments. This established that a cdc25-related fragment different from that previously described had been cloned.

The insert from one PCR-derived clone (p5wl) was used to screen a human cDNA library in the ggt10 vector. From approximately 10^6 plaques screened, nine positive clones were obtained. Eight corresponded to the originally cloned PCR product used as the hybridization probe. This is referred to as cdc25 A. A second cdc25 clone, isolated by using low stringency hybridization with pSwl, was called cdc25 B. The longest cDNA clones of cdc25 A and B were subjected to nucleotide sequencing. The region of each that contains the open reading frame is shown in Figure 1. cdc25 A and cdc25 B are predicted to encode proteins of 526 and 566 amino acids respectively. The calculated isoelectric point for cdc25 A is 6.3, and for cdc25 B is 5.9. Both genes have an initiation codon flanked by a Kozak consensus sequence (PuCC/GATGG) (Kozak, M. Cell 44:283-292 (1986)).

Comparison of the amino acid sequence of cdc25 A and cdc25 B and the GenBank data base (release 67) revealed homology to the previously described human cdc25 (Sadhu, K. et al., Proc Natl Acad. Sci.USA 87: 5139-5143 (1990)), referred to herein as cdc25 C. This comparison showed that there is 48% identity in the 273 C-terminal region between cdc25 C and A, and 43% identity between C and B. (Figure 2). Drosophila string shares 34.5% identity to cdc25 A in a 362 amino acid region, and 43.9% identify to cdc25 B in a 269 amino acid region (Figure 2). S.pombe cdc25+ is also related to both cdc25 A and B, though at a

lesser level (Figure 2). Human cdc25 A and cdc25 B proteins also contain conserved amino acids that characterize the "cdc25-box", particularly those in the region potentially involved in cdc25 catalytic activity (L/VFHCEXXXXR) (SEQ ID NO. 8) (Moreno, S. and P. Nurse, Nature 351:194 (1991); Gautier, J. and J. Maller, EMBO J. 10:177-182 (1991)). All known human cdc25 homologues contain a stretch of 15 identical amino acids in this region, called the highly conserved region (SEQ ID NO. 9) (Figure 2). Interestingly, the overall similarity between different human cdc25 proteins does not greatly exceed that between humans and such evolutionarily distinct species as Drosophila.

EXAMPLE 2 Assessment of the Functional Relationship Between Proteins Encoded by Human cdc25 A, cdc25B and Fission Yeast cdc25

To test whether the human cdc25 A and B genes do indeed encode proteins that are functionally related to fission yeast cdc25, the human genes were subcloned into the S. pombe autonomously-replicating expression vector, pARTN (carrying the LEU2 marker under the control of the constitutive alcohol dehydrogenase promoter, as described in experimental procedures). After introduction of the plasmids into an H+ cdc25-22 leul-32 strain, transformants were plated on media either lacking or containing leucine at a permissive (26°C) or restrictive temperature (36°C). Both human cDNAs could efficiently rescue the temperature-sensitive mutation of the cdc25 gene. Cells bearing human cDNAs were able to form single colonies with a growth rate similar to wild-type cells. Microscopic examination revealed that cells transformed with either gene were slightly "wee", a phenotype previously observed in fission yeast transformed with the wild-type cdc25+ gene on the

same type of vector (Russell, P. and P. Nurse, Cell 45:145-153 (1986)).

EXAMPLE 3 Demonstration That cdc25 A Acts in Mitosis

In order to test the role of cdc25 A, we prepared polyclonal antibodies against a peptide corresponding to an internal region of the cdc25 A protein (see Experimental Procedures). This serum was used to precipitate ^{35}S-methionine labeled HeLa proteins. A protein of 75kD was specifically precipitated in the absence, but not the presence, of competing antigenic peptide (data not shown). Stringent detergent conditions were used that abolish interactions with cdc2 and cyclin. This molecular weight is higher than predicted from the amino acid sequence of the gene; however, *in vitro* translation of the cdc25 A clone also yielded a protein of 75 kD (not shown). To test whether this protein might activate inactive cyclin B/cdc2, as described in the case of the Drosophila string protein (Kumagai, A. and W.G. Dunphy, Cell 64:903-914 (1991)) and also in the case of human cdc25 C (Strausfeld, U. *et al.*, Nature 351:242-245 (1991)), HeLa cell cdc25 A was eluted from an immunocomplex under conditions of low pH (see Experimental Procedures). The eluted protein did not possess any histone kinase activity (data not shown). This protein was mixed with cdc2/cyclin B, prepared by p13-Sepharose precipitation of an extract of HeLa cells that had been arrested in hydroxyurea and released for four hours (see Experimental Procedures). Under these conditions, the cdc2/cyclin B is relatively inactive as a histone kinase, unless the eluted cdc25 A protein is added (data not shown).

To address the function of cdc25 A protein in human cells, affinity-purified anti-peptide antibodies were microinjected into actively proliferating HeLa cells (see

Experimental Procedures). Islands of injected cells were photographed at 8, 12, 18 and 24 hours, and in another set of experiments at 8, 12, 18, 24 and 36 hours. In some cases, cells were stained with anti-rabbit IgG to confirm the success of the anti-cdc25 antibody microinjection. Analysis of the photographs in three such independent experiments led to the conclusion that the antibodies prevent cells from dividing (Figures 3A, 3B). The percentage of cells in mitosis (defined as rounded-up mitotic figures) increased progressively following microinjection of anti-cdc25A, but not following a control serum (Figure 3A). The cell number in each injected island increased in the case of control serum, but gradually declined in the experimental. This is attributed to the failure of cells to divide, coupled with their eventual death (visualized as shrivelled rounded cells) and their dissociation from the surface of the culture plate. In fission yeast, loss of cdc25 function causes cells to arrest in G2, rather than in mid-mitosis as in the present experiment. This, on the basis of sequence homology, function in fission yeast, and, in the case of cdc25 A, functional studies in human cells, the newly-identified human proteins can be classified as relatives of cdc25.

EXAMPLE 4 Activation of cdc25 by B-type Cyclin

In order to study the regulation of the cdc25 phosphatase activity in vitro, human cdc25 A and B were expressed in bacteria as fusion proteins with glutathione-S-transferase (GST, Smith, D.B. and K.S. Johnson, Gene 67:31-40 (1988)). Fusion proteins with a relative molecular weight of 90 kD (cdc25 A) and 88 kD (cdc25 B) were isolated by affinity chromatography on glutathione-Sepharose beads as described (Smith D.B. and K.S. Johnson, Gene 67:31-40 (1988)). Human cyclins A, B1, B2 and murine

D1 (CYLI, Matsushime, H. et al., Cell 65:701-713 (1991)) were expressed as fusion proteins with GST; purified proteins were obtained by the same method.

To investigate the potential regulation of cdc25 activity by cyclin, it was necessary to find a substrate that bore no conceivable relationship to cdc2, the presumed physiological substrate of the phosphatase. cdc2 binds to cyclin (Draetta, G. et al., Cell 56:829-838 (1989)) and thus addition of cyclin to a reaction containing cdc2 as the substrate would probably result in alteration of the target substrate and confuse the interpretation of any observed effect. For this reason a substrate often employed in tyrosine phosphatase studies, namely reduced, carboxamidomethylated and maleylated lysozyme (RCML) was used. (Tonks, N.K. et al., J. Biol. Chem. 263:6731-6737 (1988)). This substrate was labelled on tyrosine residues with ^{32}p and kindly provided by N. Tonks.

Cyclins purified from bacteria displayed no phosphatase activity against RCML (Figure 4A). However, cdc25 A had an endogenous tyrosine phosphatase activity (Figure 4A; see also Experimental Procedures), that is linear for at least 30 minutes (data not shown). If it is assumed that all the bacterial cdc25 protein is equally catalytically active, we can calculate that each molecule of cdc25 releases approximately one phosphate per 10 minutes. Addition of cyclin A or D to the reaction mixture had neither stimulatory nor inhibitory effect on the endogenous activity of cdc25 A at any concentration tested (Figure 4A). However, similar addition of either cyclin B1 or B2 had an approximately four-fold stimulatory effect (Figure 4A). In the preceding experiments, 10 pmoles of cyclin and cdc25 protein were used in the reaction mixture. The dependency of the activation of cdc25 on the amount of added cyclin B1 was also

investigated. The assay was performed either without cyclin or with the addition of 1, 2, 5, 10, or 20 pmoles of the cyclin B1. The reaction was performed for 20 min, and terminated by the addition of trichloroacetic acid (TCA). Activation was observed to plateau at 10 pmoles of added cyclin B1 and no further effect was detected at higher concentrations (Figure 5). Thus, under these experimental conditions, maximal activation of cdc25 is achieved by stoichiometric addition of cyclin B.

Whether the same stimulatory effect of B-type cyclins on the catalytic activity of cdc25 A could be detected was tested using other substrates including p-nitrophenylphosphate (PNPP), another frequently used PTPase substrate (Tonks, N.K. <u>et al</u>., <u>J. Biol. Chem. 263</u>:6731-6737 (1988); Guan, K. <u>et al</u>., <u>Nature</u> <u>350</u>:359-362 (1991); Dunphy, W.G. and A. Kumagai, <u>Cell 67</u>:189-196 (1991)) and the 18-mer peptide corresponding to the N-terminal region of the cdc2 protein surrounding Tyr15 (see Experimental Procedures). In the first case, the catalytic rate for cdc25 A was activated four to five-fold, specifically in the presence of cyclin B (Figure 4C). 50 pmoles of cyclin and cdc25 protein were used in this PNPP assay. When the 18-mer peptide was used, similar levels of cdc25 A activation by B cyclins were detected (Figure 4B). 10 pmoles of cdc25 protein and cyclin were used in this experiment.

<u>EXAMPLE 5</u> <u>Cyclin B1/cdc2 Interacts with cdc25A</u>

To investigate the possibility of stable interaction between cdc25 and cyclin, as predicted from the data on the activation of the cdc25 A phosphatase activity and additional work described in Example 4, immunoprecipitates with the cdc25 A anti-peptide antibody described above were prepared. In this case, immunoprecipitations were performed under conditions favorable for retention of

cdc25 protein complexes (see Experimental Procedures). Immunoprecipitates were probed with anti-cyclin B1 antibody (kindly provided by J. Pines) or the anti-cdc2 antibody (G6), prepared against C-terminal peptide of the cdc2 (Draetta, G. et al., Nature 336:738-744 (1988)). Clear signals were detected in both cases, indicating that human cdc25 protein is present in a complex with both cyclin B1 and cdc2 (data not shown).

EXAMPLE 6 Selective Inhibition by p13

p13 is an essential subunit of the cdc2 protein kinase. An excess of p13 can, however, inhibit activation of pre-MPF. To test whether p13 could directly influence the phosphatase activity of either of the human cdc25 proteins, the phosphatase assay as described in Examples 4 and 5 was performed with the addition of a final concentration of 25 mM, with or without 0.5 mM (10 pmoles) cyclin B1. In the case of cdc25 A, a 2-3-fold inhibition of the endogenous phosphatase activity was observed by adding p13 at 25 mM (Figure 6). This concentration is far higher than that of the cdc25 protein itself (0.3 mM) but is similar to that required to prevent pre-MPF activation in vivo or in vitro (Dunphy, W. et al., Cell 54:423-431 (1988); Dunphy, W. and J.W. Newport, Cell 58:181-431 (1989)). Addition of cyclin B1 in an equimolar concentration to the phosphatase was able to substantially negate the inhibitory effect of p13, causing an eight-fold activation (Figure 6). The behavior of cdc25 B was quite different. In preliminary experiments, it was found that the pH optimum for this phosphatase is 8.8 (as opposed to 8.0 for cdc25 A). At this pH, cyclin B1 could activate cdc25 B to a similar degree to cdc25 A. However, no effect of p13 on the activity of cdc25 B was observed either in the presence or absence of cyclin B (Figure 6).

EXPERIMENTAL PROCEDURES

The following experimental procedures were used in the work described in Examples 7-13.

Oocyte and Extract Preparation

Xenopus laevis propbase oocytes were prepared as described (Jessus, C. et al., FEBS Letters 266: 4-8 (1987)) and were induced to mature by 1 mM progesterone. Xenopus metaphase unfertilized eggs were activated in 1 mM HEPES pH7.4, 8.8 mH NaCl, 10 mg $CaCl_2$, 33 mH $Ca(NO_3)_2$, 0.1 mH KCl, 82 mM $MgSO_4$, 5 mg/ml Ca^{2+}-ionophore A-23187 (Sigma) and 100 mg/ml cycloheximide (Sigma). After 40 min, eggs were either homogenized and referred as "activated eggs", or washed, transferred to incubation buffer (Jessus, C. et al., FEBS Letters 266:4-8 (1987)) and homogenized at different times. To prepare extracts, oocytes were washed extensively in extraction buffer EB (Cyert, H.S. and M.W. Kirschner, Cell 53:185-195 (1988)) 80 mM b-glycerophosphate pH7.3, 20 mM EGTA, 15 mM $MgCl_2$, 1mM DTT), then lysed at 4°C in one volume of EB with protease inhibitors (25 mg/ml leupeptin, 25 mg/ml aprotinin, 1 mM benzamidine, 10 mg/ml pepstatin, 10 mg/ml soybean trypsin inhibitor and 1 mM PMSF) and centrifuged for 1 h at 100,000xg at 4°C. The supernatant was then filtered through 0.22 mm Millex-GV filters (Millipore) before use.

Prpearation and Use of p13-Sepharose Beads

P13 was purified and conjugated to sepharose as previously described (Brizuela, L. et al., EMBO J. 6:3507-3514 (1987)). After preincubation for 1 h with Sepharose CL-6B and centrifugation to remove non-specific binding, 100 ml of oocyte extracts were incubated for 90 rain at 4°C under constant rotation with 400 ml of EB plus protease inhibitors and 20 ml of p13-Sepharose beads. p13-Sepharose beads were further washed three times in EB,

then either resuspended in 80 ml of Laemmli sample buffer (Laemmli, U.K., Nature 227:680-685 (1970)) and boiled for 3 min, or immediately used for histone HI kinase assay.

Preparation of 0-33% Ammonium Sulfate Extracts

Prophase oocytes were rinsed extensively in EB, then lysed in one volume of EB with protease inhibitors at 4°C and centrifuged at 41,000 rpm for 90 rain at 4oC in Ti.41 rotor (Beckman). The supernatant was removed and filtered through 0.22 mm Millex-GV filters (Millipore). Ammonium sulfate fractionation was carried out by addition of 0.5 volume of a saturated solution of ammonium sulfate in EB to the extract, incubation on ice for 45 min, centrifugation at 41,000 rpm for 90 min at 4°C and resuspension of the pellet in one-tenth of the initial volume to a final protein concentration of 15 mg/ml, as determined with the BioRad protein assay kit with q-globulin as the standard. This extract (termed 0-33% fraction) was dialyzed for 2 h at 4oC against EB in the presence of protease inhibitors and stored at -70oC until use. For activation, extracts were incubated at room temperature with 1 mM ATP, 50 mg/ml creatine phosphokinase (Boehringer Mannheim) and 10 mM creatine phosphate (Boehringer Mannheim).

Antibodies

Fission yeast cdc25 protein was produced in Escherischia coli expressing the full-length protein (Ducommun, B. et al., Biochem. Biophys. Res. Comm. 167:301-309 (1990)). Bacterially produced cdc25 protein was purified and solubilized as described by Kumagai and Dunphy (Kumagai, A. and W.G. Dunphy, Cell 64:903-914 (1991)). To purify B1 anti-cdc25 serum (Ducommun, B. et al., Biochem. Biophys. Res. Comm. 167:301-309 (1990)), bacterially expressed cdc25 protein was subjected to

SDS-polyacrylamide electrophoresis and extracted by incubation of the excised gel pieces in PBS (phosphate saline buffer) (0.1% SDS 0.5% b-mercaptoethanol) at 37oC for 16 h. After centrifugation, the protein was concentrated on Centricon-10 microconcentrators (Amicon) and incubated with nitrocellulose (0.45 mM; Schleicher and Schuell) for 3 h at room temperature. After three ten minute washes in PBS (0.1% SDS), filters were blocked for 4 h at room temperature with PBS containing 1.5% BSA (bovine serum albumin, Boehringer Mannheim) and 0.5% Tween-20. After three ten-minute washes in PBS (0.1% SDS), filters were incubated at room temperature for 16 h with B1 anti-cdc25 serum (Ducommun, B. et al. Biophys. Res. Comm. 167:301-309 (1990)), and diluted four times in PBS 1.5% BSA. Filters were then washed three times for 10 rain with PBS (0.1% Tween-20) and once for 10 min with PBS. Purified anti-cdc25 antibody was eluted with 1 ml of 100 mM glycine pH2.5, and 200 ml of 1 M TRIS pH8.0 was added after 1 min. After addition of 300 ml of PBS (10% BSA, 0.5% NaN_3), the purified antibody was stored at 4oC until use. For some control experiments, the purified antibody was preadsorbed overnight at 4oC with 10 mg/ml purified bacterially expressed yeast cdc25 protein before Western blotting.

Anti-B2 cyclin antibody was a gift from J. Gautier (rabbit polyclonal purified antibody directed against Xenopus cyclin B2; Gautier, J. et al., Cell 60:487-494 (1990); Gautier, J. and J. Maller, EMBO J. 10:177-182 (1991)). Anti-cdc2 antibody was a rabbit polyclonal purified antibody directed against thr full-length *Schizosaccharomyces pombe* cdc2 (Draetta G. et al., Cell 50:319-325 (1987)). Anti-phosphotyrosine antibody was a mouse IgG monoclonal antibody (Ab-1, Oncogene Science). The sensitivity of this anti-phosphotyrosine antibody ought to have been be sufficient to allow the detection of

phosphotyrosine in the cdc25-associated cdc2, since a comparable amount of prophase cdc2 was easily recognized. Therefore, the absence of signal observed in metaphase cdc2 bound to cdc25 suggested that this population of cdc2 was not phosphorylated on tyrosine.

Immunoprecipitation and Western Blot Analysis

100 ml of oocyte extracts in EB were mixed with 400 ml of Eb and incubated for 1 h at 4°C with 30 ml of protein A-agarose beads (Pierce). Anti-cdc25 antibody (dilution 1:100), anti-cyclin B2 antibody (dilution 1:50) or anti-cdc2 antibody (dilution 1:500) were then added to the supernatant and after a 5h incubation at 4°C, 30 ml of protein A-agarose beads were added. After an additional 1 h incubation at 4°C, the beads were either washed four times in EB and then eluted by boiling for 30 min in 80 ml Laemmli sample buffer or resuspended in kinase buffer (50 mM TRIS pH7.4, 10 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT) for a subsequent histone HI kinase assay.

To elute Xenopus cdc25 protein from immunoprecipitates, immunocomplexes were resuspended in 250 ml of 100 mM glycine pH2.5. After a 2 min stirring, 50 ml of 1 M TRIS pH8.0 was added. The supernatant was recovered, concentrated on Centricon-10 microconcentrators (Amicon) and bovine serum albumine was added to a final concentration of 0.1%.

Electrophoresis and Western blot analysis with anti-cdc25 antibody (dilution 1:500), anti-cyclin B2 antibody (dilution 1:100) or anti-cdc2 antibody (dilution 1:000) were performed as previously described (Booher, R.N. et al., Cell 58:584-497 (1989)). By scanning immunoblots of initial extracts before anti-cdc25 immunoprecipitation, extracts after anti-cdc25 immunoprecipitation and anti-cdc25 immunoprecipitates

(FujiX Bas 2000 Image Analyzer), it was we estimated that 70% of the full cellular amount of cdc25 was immunoprecipitated by the anti-cdc25 antibody. In a parallel way, the amount of p72 associated with cdc2 or cyclin B2 in immunoprecipitates was quantified by Image Analyzer (FujiX Bas 2000), by using anti-cdc25 immunoblots of crude extracts as a reference of the full cellular amount of cdc25. 20% of the total cellular amount of cdc25 was found either in anti-cdc2 immunoprecipitates or in anti-cyclin B2 immunoprecipitates. To quantify the amount of cdc2 or cyclin B2 associated with cdc25, equal amounts of oocyte extracts (from 10 oocytes, equivalent to 200 mg of proteins) were either precipitated on p13-Sepharose or immunoprecipitated with anti-cdc25 antibody. p13-sepharose beads completely clear the extract of cdc2 and cyclin B2 as ascertained by Western blotting (data not shown) and, therefore, p13-precipitate represents the full cellular amount of cdc2 and cyclin B2. On the other hand, the anti-cdc25 immunoprecipitate contains only the cdc2 and the cyclin B2 that are associated with p72. Both p13-precipitates and anti-cdc25 immunoprecipitates (each the equivalent of 10 oocytes) were loaded on the same electrophoresis gel and blotted with the anti-cdc2 antibody or the anti-cyclin B2 antibody. The relative amounts of cdc2 and cyclin B2 detected in both extracts were determined by PhosphorImager (Molecular Dynamics) or Image Analyzer (FujiX Bas 2000). The amount of cdc2 present in p13-Sepharose precipitate is 20-fold higher than that detected in the anti-cdc25 immunoprecipitate. Thus, 5% of the total cdc2 is associated with p72. The amount of cyclin B2 present in p13-Sepharose precipitates is 6-fold higher than that detected in the anti-cdc25 immunoprecipitate. Thus, 17% of the total cyclin B2 is associated with p72.

Histone H1 Kinase Assay

p13-precipitates or immunocomplexes were washed three times in kinase buffer and then resuspended in 50 ml of kinase buffer containing 0.2 mg/ml histone H1 (Boehringer Mannheim), 50 mM ATP and 1 mCi[q^{32}P]ATP (PB.10168, Amersham). After a 30 min incubation at 30°C, the reactions were terminated by the addition of 30 ml Laemmli sample buffer (Laemmli, U.K., Nature 227:680-685 (1970)). Samples were electrophoresed on a 12% polyacrylamide gel. After staining with coomassie blue and autoradiography, ^{32}p incorporation into histone H1 was quantified by scintillation counting of excised gel pieces.

Protein samples from the 0-33% fraction (in a volume of 10 ml of EB) were mixed on ice with 40 ml of kinase buffer containing 0.2 mg/ml histone H1, 25 mM ATP, 2 m Ci[q^{32}P]ATP and 10 mM cAMP dependent protein kinase inhibitor peptide (P3294, Sigma). After incubation for 10 min at 30°C, samples were treated as previously described.

EXAMPLE 7 cdc25 Protein in Xenopus Oocytes

An anti-cdc25 serum directed against fission yeast cdc25 was used to determine whether a cdc25 protein is present in Xenopus oocytes. This serum, previously referred to as B1 (Ducommun, B. et al., Biochem. Biophys. Res. Comm. 167:301-309 (1990)), was affinity purified as described in the Experimental Procedures. It recognizes the full-length yeast cdc25 product expressed in *E. coli* but no signal is detectable in an *E. coli* lysate before transcriptional cdc25 induction of cdc25 (Ducommun, B. et al., Biochem. Biophys. Res. Comm. 167:301-309 (1990)).

Extracts were prepared from the following cells: meiotic prophase-blocked oocytes; meiotic metaphase unfertilized eggs; eggs activated in the presence of cycloheximide, that therefore lack cyclin and are blocked in an interphase state (Murray, A.W. and Kirschner, M.

Nature 339:275-280 (1989)); and eggs after 120 min of activation (after completion of the first MPF cycle). These extracts were probed with the affinity-purified serum in an immunoblot. A 72 kD polypeptide was detected in each sample. No signal was detected using the same procedure but substituting preimmune serum or purified antibody preadsorbed with soluble bacterially-expressed yeast cdc25 protein for the affinity-purified serum (data not shown). Furthermore, two other purified polyclonal antibodies directed against the yeast cdc25 protein were able to recognize the same 72 kD protein from Xenopus extracts. (Ducommun, B. et al., Biochem. Biophys. Res. Comm. 167:301-309 (1990)).

To test whether the 72 kD species might be immunoprecipitated by the anti-cdc25 antibody, extracts from prophase oocytes, metaphase unfertlllzed eggs and interphase eggs activated in the presence of cycloheximide were precipitated with the purified anti-cdc25 antibody and probed with the same purified serum in immunoblots. Again, a protein of 72 kD was speclfically detected by the cdc25 antibody (data not shown). In contrast, no signal was detected when the same procedure was used in the absence of Xenopus extract, formally demonstrating that the 72 kD protein observed in the immunoprecipitates is not due to the presence of cdc25 protein in the antibody preparation (a contamination that could occur during immuno-affinity purification of the antibody).

To obtain soluble 72 kD polypeptide, proteins were eluted from anti-cdc25 immunoprecipitates at low pH (see Experimental Procedures) and the amount of 72kD protein was determined by immunoblotting with the cdc25 antibody. Again, the same level of 72 kD protein was found in prophase oocytes, metaphase unfertillzed eggs, interphase-blocked activated eggs and eggs after the completion of the first MPF cycle (data not shown).

EXAMPLE 8 Demonstration That cdc25 Activates the M-phase Kinase

Human and Drosophilia cdc25 proteins are able to trigger activation of cdc2/cyclin B *in vitro* (Kumagai, A. and W.G. Dunphy, Cell 64:903-914 (1991); Strausfeld, U. *et al.*, Nature 351:242-245 (1991)) by dephosphorylating cdc2 (Dunphy, W.G. and A. Kumagai, Cell 67:189-196 (1991); Gautier, J. *et al.*, Cell 67:197-211 (1991)). As a further test that the anti-cdc25 antibody recognized Xenopus cdc25, it was investigated whether the 72 kD protein eluted from immunocomplexes could stimulate inactive cdc2. To prepare inactive enzyme from prophase oocytes p13-Sepharose beads were used. Xenopus cdc2 protein binds strongly and quantitatively to fission yeast p13 (Dunphy, W. *et al.*, Cell 54:423-431 (1988)). The p13-Sepharose bound cyclin B/cdc2 complex from prophase oocytes has a low histone H1 kinase activity. Protein eluted from anti-cdc25 immunoprecipitates of either prophase oocytes or metaphase unfertilized eggs was added to inactive prophase p13-bound cdc2. After a 30 min preincubation at 30°C in the presence of cdc25-immunocomplex eluates, the p13-precipitate was extensively washed and then assayed for histone H1 kinase activity. Both prophase and metaphase cdc25 stimulated histone H1 kinase activity 12-fold. The possibility that some of the histone HI kinase activity present in the anti-cdc25 immunocomplexes (see below) might be responsible for this increase of kinase activity was eliminated. First, the p13-Sepharose precipitate was extensively washed after preincubation with the immunoeluted material, and before assay of kinase activity. Second, the histone H1 kinase activity found associated with the eluted metaphase proteins was insufficient to account for the observed 12-fold stimulation of the p13-bound enzyme (approximately 500 units of final activity). Third, the prophase

immuno-eluted material was also able to activate cdc2, although it did not contain any kinase activity (data not shown). It was therefore concluded that an active Xenopus cdc25 protein was precipitated by the affinity-purified anti-cdc25 antibody from both prophase oocytes and metaphase eggs. It is surprising that active p72 could be extracted from Xenopus oocytes in which cdc2/cyclin B is inactive and tyrosine phosphorylated.

It was also tested whether p72 from either prophase oocytes or metaphase unfertilized eggs could affect the activity of either fully activated cdc2/cyclin from metaphase unfertilized eggs or cdc2 that is inactive in the absence of cyclin (material extracted from eggs activated in the presence of cycloheximide). In neither case did p72 have any effect on the histone H1 kinase activity of cdc2 (data not shown). The 135 units of activity found in one sample of activated eggs is probably due to the basal activity of cdc2 from activated eggs (66 units) combined with the kinase activity associated with metaphase cdc25 and therefore does not represent a real stimulation of cdc2. It was concluded that p72 only acts on the tyrosine phosphorylated enzyme.

<u>Example 9</u> <u>Demonstration That Activation of pre-MPF Requires cdc25</u>

Xenopus prophase oocytes contain an inactive form of MPF that can be activated by a post-translational mechanism both <u>in vivo</u> (Wasserman, W. and Y. Masui, <u>Exp. Cell. Res. 91</u>:381-388 (197S); Gerhart, J. <u>et al</u>., <u>J. Cell Biol. 98</u>:1247-1255 (1984)) and <u>in vitro</u> (Cyert, M.S. and M.W. Kirschner, <u>Cell 53</u>: 185-195 (1988); Dunphy, W.G. and J.W. Newport, <u>Cell 58</u>: 181-191 (1989)). Addition of an ATP-regenerating system to a prophase oocyte extract (33% ammonium sulfate precipitated fraction) is sufficient to induce tyrosine dephosphorylation of cdc2 and stimulation

of its latent activity (Cyert, M.S. and M.W. Kirschner, Cell 53: 185-195 (1988); Dunphy, W.G. and J.P. Newport, Cell 58: 181-191 (1989)). In order to determine if endogenous p72 was required for this activation process, the effect of adding anti-cdc25 antibody to the 0-33% ammonium sulfate fraction from phophase oosytes was explored. 200 ml of the 0-33% ammonium sulfate fraction of high speed extract of phophase oocytes was incubed for 15 min at 40°C. At 0 min, samples were transferred to room temperature, and 1 mM ATP, 10 mM creative phosphase and 50 mg/ml creative phosphokinase were added. Following the addition of this ATP-regenerating system to the extract, the histone H1 kinase was rapidly activated (Fig. 8). By contrast, a 15 min preincubation of the extract with anti-cdc25 antibody resulted in a prolonged inhibition of the activation process. Addition of the preimmune anti-cdc25 serum had no effect (Fig. 8). This result suggests that the endogenous p72 is required for histone H1 kinase activation and is inactivated after immunocomplexing with the antibody. It was further found that bacterially-expressed cdc25 protein at 100 mg/ml, when added at 60 minutes, can overcome the inhibition caused by the anti-cdc25 antibody (Fig. 8), indicating that the antibody acts specifically on the endogenous cdc25 protein.

EXAMPLE 10 Demonstration of an Association Between cdc25 and cdc2 at M-phase

To investigate further the mechanism of cdc2 activation by cdc25, the possibility that cdc25 might directly associate with the M-phase enzyme was tested. Extracts of either prophase oocytes, metaphase unfertilized eggs or activated eggs were immunoprecipitated with an anti-cdc2 antibody and probed with the same anti-cdc2 antibody. As expected, a strong signal was obtained (data not shown).

Since the anti-cdc2 antibody recognized a single 34 kD band, it was assumed that this antibody does not react with cdk2, a 32 kD cdc2-like protein encoded by the Xenopus Egl gene (Paris, J. et al., Proc. Natl. Acad. Sci. USA 88:1039-1043 (1991)). Similar anti-cdc2 immunoprecipitates were probed with the purified anti-cdc25 antibody. A 72 kd band was observed in the metaphase unfertilized eggs, but not in the resting prophase oocytes or in the eggs activated in the presence of cycloheximide. In a control experiment in which the purified anti-cdc25 antibody was preadsorbed with bacterially expressed cdc25 protein before immunoblotting, no signal was detected. These results indicate that cdc25 stably associates with cdc2 at M-phase.

To further test the existence of an association between cdc2 and cdc25 the converse experiment was also performed. Cdc25 was immunoprecipitated from prophase oocytes, metaphase unfertilized eggs and activated eggs using the purified anti-cdc25 antibody. An equal amount of cdc25 was precipitated in each case (data not shown). The anti-cdc25 immunoprecipitates were then probed with the anti-cdc2 antibody. A 34 kD protein was detected in the metaphase unfertilized eggs, but not in the prophase oocytes or in the activated eggs (data not shown). To confirm that the 34 kD protein detected in this experiment was indeed cdc2, prophase oocyte, metaphase unfertilized egg and activated egg extracts were first depleted of the cdc2/cyclin B complex by preincubation with p13-Sepharose and then immunoprecipitated with the purified anti-cdc25 antibody. Immunoblotting these immunocomplexes with anti-cdc2 antibody revealed complete depletion of the 24 kD protein (data not shown). Therefore, it was concluded that the 34 kD protein was cdc2. Moreover, cdc2, which is present at the same level in prophase oocytes, metaphase eggs and interphase eggs, was not recognized in an

immunoblot by the purified anti-cdc25 antibody, indicating that there was no cross-reactivity between cdc2 and the anti-cdc25 antibody. By quantifying the signal in immunoblots (see Experimental Procedures), it was estimated that the amount of cdc2 present in anti-cdc25 immunoprecipitates represented approximately 5% of the total cellular cdc2 at metaphase and that the amount of cdc25 present in anti-cdc2 immunoprecipitates represented 20% of the cellular content of cdc25.

EXAMPLE 11 Demonstration That Cyclin B is Associated with cdc2 and cdc25 at M-Phase

Since the active cdc2 from M-phase is associated with cyclin (Brizuela, L. *et al.*., *Proc. Natl. Acad. Sci. USA* *86*:4362-4366 (1989); Draetta, G. *et al.*, *Cell* *56*:829-838 (1989); Gautier, J. *et al.*, *Cell 60*:487-494 (1990)), it was further investigated whether cyclin B is present in association with cdc2 and cdc25 at M-phase. Extracts of either prophase oocytes, metaphase unfertilized eggs or activated eggs were precipitated with p13-Sepharose and probed with an anti-cyclin B2 antibody. Cyclin B2 was present in both prophase oocytes and metaphase unfertilized eggs (data not shown). As already noted (Gautier, J. and J. Maller, *EMBO J.* 10:177-182 (1991); Kobayashi, A.H. *et al.*, *J. Cell Biol.* *114*:755-765 (1991)), two immunoreactive bands of cyclin B2 are detectable, of which the upper band was a phosphorylated form appearing during meiotic maturation. In contrast, cyclin B2 was not detectable in eggs activated in the presence of cycloheximide (data not shown). The same extracts were immunoprecipitated with the anti-cyclin B2 antibody and then probed with the purified anti-cdc25 antibody. The 72 kD protein was detected in association with cyclin B2 in the metaphase eggs but not in the prophase oocytes or in the interphase eggs (data not

shown). The converse experiment was then performed. The three types of cell extracts were immunoprecipitated with the purified anti-cdc25 antibody and probed with the anti-cyclin B2 antibody. Cyclin B2 was associated with cdc25 in metaphase unfertilized eggs, but not in resting prophase oocytes or activated eggs (data not shown). The phosphorylated form of cyclin B2 was predominantly associated with cdc25. As a control experiment, prophase oocyte, metaphase egg and activated egg extracts were first depleted of cdc2/cyclin B by incubation with p13-Sepharose and then immunoprecipitated with the anti-cdc25 antibody. No signal was detected after probing these extracts with the anti-cyclin B2 antibody, indicating that the 51 kD band previously detected was indeed cyclin (data not shown). It was therefore concluded that cdc25 binds to the cyclin B/cdc2 complex at metaphase. The amount of cdc25 present in anti-cyclin B2 immunoprecipitates was estimated to be the same as the proportion of cdc25 previously found in association with cdc2 (20% of the full cellular content of cdc25). In contrast, it was determined that cdc25-associated cyclin B2 represents 17% of the total population of cyclin B2, which is a higher percentage than the amount of cdc25-associated cdc2 (5%).

<u>EXAMPLE 12</u> <u>M-phase Kinase Associated with cdc25 is Active</u>

At metaphase, cdc2 is predominantly tyrosine dephosphorylated and active as a histone H1 kinase. Since cdc2 is associated with cdc25 only at metaphase, the tyrosine phosphorylation state and the kinase activity of the complexed cdc2 were investigated. By immunoblotting p13-Sepharose precipitates with an anti-phosphotyrosine antibody, it was confirmed that cdc2 was heavily tyrosine phosphorylated in prophase oocytes and substantially

dephosphorylated in metaphase unfertilized eggs, although different batches of metaphase eggs display a somewhat different degree of cdc2 tyrosine dephosphorylation, as previously demonstrated (Dunphy, W.G. and J.W. Newport, Cell 58:181-431 (1989); Jessus, C. et al., FEBS Letters 266:4-8 (1990). No tyrosine phosphorylation of cdc2 could be detected in eggs that were activated in the presence of cycloheximide and thus lack cyclin B. (See also Solomon, M.J. et al., Cell 63:1013-1024 (1991)). When anti-cdc25 immunocomplexes from prophase oocytes, metaphase unfertilized eggs or activated eggs were probed with the same anti-phosphotyrosine antibody, no phosphotyrosine-containing proteins were detected, despite the presence of abundant cdc2 in the immunocomplex from metaphase unfertilized eggs (data not shown). If the cdc25-associated cdc2 were substantially tyrosine phosphorylated, a signal of sufficient strength would have developed in the immunoblot. This result suggested that the fraction of cdc2 associated with cdc25 in metaphase unfertilized eggs was likely to be active as a histone H1 kinase. This was found to be true: the kinase activity in p13-Sepharose precipitates was very low in prophase oocytes, was increased 31-fold in metaphase unfertilized eggs and declined during activation in the presence of cycloheximide. Histone H1 kinase activity was detected in anti-cdc25 immunoprecipitates from metaphase eggs. The activity detected in anti-cdc25 immunoprecipitates from prophase oocytes and activated eggs was comparable to the background levels (data not shown), indicating that no cdc2 kinase was present in these extracts. By comparing the relative metaphase kinase activity in P-13 Sepharose precipitates and anti-cdc25 immunoprecipitates (approximately 20-fold different) it was found that the specific activity of cdc2 was essentially identical in each sample.

EXAMPLE 13 Association Between cdc2/cyclin B and cdc25 is Periodic

The abundance of the Xenopus cdc25 protein appears not to vary during meiotic maturation or in the first embryonic cycle (data not shown). However, the protein was only found in association with cdc2 and cyclin B in metaphase unfertilized eggs. To investigate this more closely, metaphase unfertilized eggs were parthenogenetically activated in the presence of CA^{2+}-ionophore and calcium, and histone H1 kinase activity was assessed in p13-Sepharose precipitates during the first 150 min. At various intervals, 100 eggs were homogenized, centrifuged, and precipitated. The histone H1 kinase activity disappeared about 20 min after activation, reappeared between 60 and 90 min at time of the first cleavage, declined again and finally peaked at time of the second mitotic cleavage (Fig. 9). Samples taken from the same cell extracts were immunoprecipitated with anti-cdc25 antibody and immunoblotted with anti-cdc2 serum to estimate the extent of association. Relative amounts of cdc2 present in the anti-cdc25 immunoprecipitates were quantified by Phosphor-Imager. The periodic interval of the association between cdc2/cyclin B complex and cdc25 was identical to the periodicity of the p13-bound enzyme activity (Fig. 9). However, a slight phase shift was noted. The association peaked slightly ahead of the overall histone H1 kinase. In repeated experiments (data not shown), the pattern of association was always the same. However, in some cases the phase shift between the histone H1 kinase activity and the association between cdc2/cyclin B and cdc25 was less obvious.

Experimental Procedures

The following materials, methods and procedures were used in carrying out the work described in Examples 14-18.

Materials and Methods

Chemicals sodium fluoride, sodium orthovanadate, nitrophenol, cis-platinum, isopropyl β-D-thiogalactopyranoside (IPTG), 1-methyladenine, dithiothreitol (DDT), EGTA, EDTA, MOPS, β-glycerophosphate, leupeptin, aprotinin, soybean trypsin inhibitor, benzamidine, histone H1 (type III-S), CNBr-activated sepharose 4B, glutathione-agarose (G 4510), glutathione (G 4251), nonidet P40 (NP40), Tris, LB Broth base, were obtained from Boehringer-Mannheim; p-nitrophenylphosphate (p-NPP) (disodium salt hexahydrate, ref. 12.886.82) was from Janssen Chimica.

[γ-^{32}P]-ATP (PB 168) and ^{125}I]-protein A (IM 144) were obtained from Amersham.

G1 anti-$p34^{cdc2}$ antibodies and anti-$p80^{cdc25}$ antibodies (directed against the cdc25C phosphatase peptide H_2N-QEGERQLREQIALLVKDMS-COOH) were kindly provided by Dr. G. Draetta (Heidelberg); anti-cyclin B^{cdc13} (starfish) antibodies were generously donated by Dr. T. Kishimoto (Tokyo); anti-phosphotyrosine antibodies were generously given by Dr. J.Y.J. Wang (La Jolla); antibodies against H_2N-VEKIGEG<u>TY</u>GVVYKARHKLS-COOH (a $p34^{cdc2}$ peptide containing the regulatory threonine-14 and tyrosine-15 residues) were kindly provided by Dr. L. Tung (Philadelphia). This last antibody does not recognize tyrosine-phosphorylated $p34^{cdc2}$ but only tyrosinedephosphorylated $p34^{cdc2}$ but only tyrosinedephosphorylated $p34^{cdc2}$.

Buffers

<u>Oocyte homogenization buffer</u> contained 60 mM β-glycerophosphate, 15 mM p-NPP, 20 mM MOPS pH 7.2, 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 0.1 mM sodium vanadate, 0.1 mM sodium fluoride, 10 µg leupeptin/ml, 10 µg aprotinin/ml, 10 µg soybean trypsin inhibitor/ml, 100 µM benzamidine. This buffer had previously been shown to

stabilize the starfish meiotic oocyte M phase-specific histone H1 kinase (Pelech, S.L. et al., Biochemistry 26:7960-7968 (1987)).

Bead buffer contained 50 mM Tris pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP40, 10 µg leupeptin/ml, 10 µg aprotinin/ml, 10 µg soybean trypsin inhibitor/ml and 100 µM benzamidine.

Tris-Buffered Saline (TBS) contained 50 mM Tris pH 7.4, 150 mM NaCl.

Phosphate-Buffered Saline (PBS) contained 9.6 mM phosphate, 2.7 mM KCl, 140 mM NaCl.

Lysis buffer contained 1% NP40, 1 mM EDTA, 1 mM DTT, 10 µg leupeptin/ml, 10 µg aprotinin/ml, 10 µg soybean trypsin inhibitor/ml and 100 µM benzamidine/ml in PBS.

Tris buffer A contained 50 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT.

Elution buffer contained 10 mM glutathione in Tris buffer A.

Preparation of G2 and M Phase Oocytes

G2 and M phase oocytes were prepared as follows: gonads were removed from mature starfish (Marthasterias glacialis), collected in Northern Brittany. They were either directly frozen in liquid nitrogen and kept at -80°C (G2 oocytes) or incubated with 10 µM 1-methyladenine in natural seawater for 10 min (M. oocytes). By that time all the oocytes had entered the M phase, although they were still in the gonads. These were then removed from the incubation medium, rapidly blotted on filter paper, directly frozen in liquid nitrogen and kept at -80°C.

Transfer buffer contained 39 mM glycine, 48 mM Tris, 0.37% SDS, 20% methanol.

Bacterial Growth and cdc25A Induction

An *E. coli* strain (BL 21(DE3)) containing a plasmid encoding the genes fusion construct of glutathione-S-transferase (GST) and human cdc25A under the control of IPTG was used (Galaktinonov, K. and D. Beach, Cell 67:1181-1194 (1991)). *E. coli* were first grown overnight at 37°C in the presence of 100 µg ampicillin/ml LB medium. Four ml of this preculture were inoculated/liter of LB containing 100 µg ampicillin/ml. Incubation was pursued at 30°C until the culture O.D. at 500 nm had reached a value between 0.8 and 1.00 (about 4-5 hrs). At this moment, 0.4 mM IPTG was added and the culture incubated at 25°C for at least 7 hours. Cells were then harvested by a 3000 g centrifugation for 15 min at 4°C. Pellets were kept frozen at -80°C until extraction.

Example 14 $p80^{cdc25}$ Controls $p34^{cdc2}$/cyclin B Activation

Inactive pre-MPF (G2) is constituted of cyclin B and $p34^{cdc2}$ phosphorylated on its threonine-14 and tyrosine-15 residues. $p80^{cdc25}$ is the phosphatase which dephosphorylats the tyrosine-15 residue, and possibly threonine-14. Its action leads to activation of the $p34^{cdc2}$/cyclin B^{cdc13} kinase responsible for induction of the G2/M transition. The interaction of these components and activation of inactive pre-MPF (G2) is represented in Figure 10. An agent to be tested for its ability to alter stimulation of kinase activity is combined with the inactive pre-MPF (G2) and the effects, if any, are determined. If an agent tested is an inhibitor, the inactive pre-MPF will not be activated.

Example 15 Production and Purification of GST cdc25A Phosphatase

A fusion construct between the glutathione-S-transferase (GST) gene and human cdc25A was built in a

plasmid vector (Galaktionov, K. and D. Beach, Cell 67:1181-1194 (1991)). Transfected and expressed in *E. coli*, it produced large amounts of the corresponding fusion protein which was purified by affinity chromatography on glutathione-agarose beads. The protocols of production, purification and assay of the GST-cdc25A phosphatase are described in detail below. Production involved culture of recombinant *E. coli* and classical induction of GST-cdc25A expression by IPTG. One-step affinity-chromatography on glutathione-agarose allowed the purification of the GST-cdc25A phosphatase. The optimum ratio of bacterial extract volume/glutathione-agarose volume was found to be 6-10 to 1. GST-cdc25A was either preserved as the bacterial pellet (very stable), the supernatant of the centrifuged bacterial extract or after affinity-purification ad in the presence of 40% glycerol (final volume).

The bacterial pellet was disrupted by sonication in lysis buffer at 4°C. The homogenate was centrifuged for 30 min at 4°C at 100,000 g; the supernatant was recentrifuged under similar conditions; the final supernatant was then immediately mixed and rotated with glutathione-agarose beads (equilibrated with lysis buffer) for 30 min at 4°C (6-10 volumes of supernatant/1 volume of packed beads). The glutathione-agarose beads were washed three times with 10 volumes of lysis buffer, followed by four washes with 10 volumes of Tris buffer A. Elution of the fusion protein was induced by 3-4 successive washes with 10 mM glutathione in Tris buffer A. The efficiency of the elution was monitored by a phosphatase assay. Active fractions were pooled and used directly or supplemented with 40% glycerol prior to storage at -80°C.

Glutathione-agarose beads easily recycled by a wash with 1 M NaCl, followed by equilibration with lysis buffer.

Example 16 Assay of the GST-cdc25A Phosphatase Activity Towards p-Nitrophenylphosphate

GST-cdc25A phosphatase activity can be very conveniently assayed using the chromogenic substrate p-nitrophenylphosphate (p-NPP). Optimal conditions for several parameters were determined with a one ml assay, as described below. Results are represented graphically in the figures: amount of GST-cdc25A phosphatase (Figure 12A), duration of assay (Figure 12B), DTT concentration (Figure 13A), p-NPP concentration (Figure 13B).

One ml assay: 100 µl of GST-cdc25A protein (diluted to an activity of ∂ OD 410 nm = 0.3/10 min) were mixed with 100 µl mM DTT (in Tris buffer A) and 700 µl of Tris buffer A. The assay was initiated by addition of 100 µl 500 mM p-NPP (in Tris buffer A). After 10 min incubation at 37°C, the assay was terminated by addition of 40 µl 5 N NaOH and transfer of the tubes to 4°C. Absorbance at 410 nm was then measured and blank values (no GST-cdc25A protein but 10 min incubation) were subtracted.

This assay was then scaled down to 200 µl and conducted semi-automatically in 96-wells microtitration plates, as described in detail below. Each well was filled with 20 µl GST-cdc25A phosphatase, 140 µl Tris buffer A, 20 µl 100 mM DTT (in Tris buffer A); after 15 min equilibration at 37°C, reaction was initiated by addition of 20 µl 500 mM p-NPP (in Tris buffer A). After 60 min incubation absorbance at 405 nm was monitored in a microplate reader; blank values (no GST-cdc25A added) were subtracted.

Microtitration plate assay: 20 µl of GST-cdc25A protein (diluted to an activity of ∂ OD 405 nm = 0.2-0.3/60 min) were mixed with 20 µl 100 mM DTT (in Tris buffer A) and 140 µl of Tris buffer A, in 96-wells microtitration plates (Corning). The plates were

preincubated at 37°C for 15 min in a Denley Wellwarm 1 microplate incubator. The assays were initiated by addition of 20 µl of 500 mM p-NPP (in Tris buffer A). After 60 min incubation at 37°C absorbance at 405 nm was measured in a bioRad microplate reader. Blank values (no CST-cdc25A protein added) were automatically subtracted.

Example 17 Tyrosine Dephosphorylation and Activation of the $p34^{cdc2}$/cyclin B^{cdc13} Kinase by the Fusion Protein GST-cdc25A

The ability of the GST-cdc25A fusion protein to dephosphorylate and activate the $p34^{cdc2}$/cyclin B^{cdc13} kinase was demonstrated. $p34^{cdc2}$/cyclin B^{cdc13} complex from G2-arrested starfish oocytes was immobilized on $p9^{CKShs1}$ agarose: it is constituted of tyrosine-phosphorylated $p34^{cdc2}$ and cyclin B^{cdc13} (Arion, L. et al., Eur. J. Biochem.: (1992); Pondaven, P. et al., Genes and Development 4:9-17 (1990)).

Treatment with purified GST-cdc25A protein induced almost complete tyrosine dephosphorylation of $p34^{cdc2}$ by the $p34^{cdc2}$ mobility shift, the loss of cross-reactivity with anti-phosphotyrosine antibodies and the appearance of cross-reactivity with an antibody directed against a $p34^{cdc2}$ peptide comprising the tyrosine-15 residue (data not shown). In addition, this tyrosine dephosphorylation lead to histone H1 kinase activation to a level close to that found in M phase oocytes (Figure 11). By these criteria, the GST-cdc25A fusion protein appears to display all the physiological enzymatic activity of cellular $p80^{cdc25}$.

Assay of $p34^{cdc2}$/Cyclin B^{cdc13} Kinase Activity

Oocyte extracts were prepared by homogenization of 1 g of G2 or M phase gonads per 2 ml homogenization buffer. After centrifugation for 10 min at 14,000 g at 4°C, the

supernatants were loaded on p9^{CKShs1}-sepharose beads prepared as described in Azzi, L. <u>et al.</u> (<u>Eur. J. Biochem.</u>: in press (1992)) (400 µl supernatant/10 µl p9^{CKShs1}-beads). The tubes were kept under constant rotation at 4°C for 30 min. After a brief centrifugation at 10,000 g and removal of the supernatant, the beads were washed three times with bead buffer and eventually exposed to purified GST-cdc25A phosphatase prior to H1 kinase assay or to immunoblotting analysis.

Histone H1 kinase assays were performed by incubation of 10 µl of packed p9^{CKShs1}-beads (loaded with G2 or M phase extracts) for 10 min at 30°C with 15 µM [γ-32P] ATP (3,000 Ci/mmol; 1 mCi/ml) in the presence of 1 mg histone II1/ml in a final volume of 40 µl. Assays were terminated by transferring the tube onto ice. After a brief centrifugation at 10,000 g, 30 µl aliquots of supernatant were spotted onto 2.5 x 3 cm pieces of Whatman P81 phosphocellulose paper, and after 20 sec, the filters were washed five times (for at least 5 min each time) in a solution of 10 ml phosphoric acid/liter of water. The wet filters were transferred into 6 ml plastic scintillation vials, 5 ml ACS (Amersham) scintillation fluid was added and the radioactivity of the samples measured in a Packard counter.

<u>Electrophoresis and Western Blotting</u>

Proteins bound to p9^{CKShs1}-sepharose beads were recovered with 50 µl 2X Laemmli sample buffer. Samples were run in 10% SDS/polyacrylamide gels. Proteins were stained with Coomassie Blue or transferred to 0.1 µm nitrocellulose sheets (Schleicher & Schull) in a Milliblot/SDE system (Millipore) for 30 min at 2.5 mA/cm^2 in transfer buffer. The filters were subsequently blocked with TBS containing 3% bovine serum albumin for 1 hr at room temperature. The filters were then incubated

overnight at 4°C with g1 anti-p34cdc2 antibodies (1:1000 dilution), anti-p34^{cdc2} peptide antibodies (1:500 dilution) or anti-phosphotyrosine antibodies (1 µg/ml). After four washes of 15 min each with TBS containing 0.2% NP40, the filters were treated with 1 µCi ^{125}I-protein A (30 mCi/mg) in 3% bovine serum albumin in TBS for 2 hr at room temperature. After four 15 min washes with 0.2% NP40 in TBS, the filters were exposed overnight to hyperfilm MP (Amersham).

Example 18 Detection of Inhibitors of Purified GST-cdc25A Phosphatase

In a series of experiments various antimitotic compounds currently used in cancer therapy were tested as potential inhibitors of the phosphatase (the Table). Most of them are reported to act as DNA damaging agents, as DNA intercalators, as topoisomerase 2 inhibitors or as compounds interfering with spindle microtubles. None of them displayed GST-cdc25A phosphatase inhibitory activity. As a positive control vanadate, a reported inhibitor of tyrosine phosphatases was also tested (Gordon, J.A., Methods in Enzymology pp. 447-482 (1991)). This compound totally inhibits the GST-cdc25A phosphatase at concentrations above 500 µM (Figure 14; I_{50} = 20 µM).

TABLE

ANTIMITOTIC COMPOUNDS TESTED AS POTENTIAL INHIBITORS OF P80^{cdc25A}

Compounds		Range of Concentration Tested
–	Actinomycin D	0.1-100 µg/ml
–	BCNU	0.1-100 µg/ml
–	Carboplatin	0.1-100 µg/ml
–	Chlormethine	0.1-100 µg/ml
–	Cis-platinum	0.1-100 µg/ml
–	Cyclophosphamide	0.1-100 µg/ml
–	Dacarbazine	0.1-100 µg/ml
–	Doxorubicin	0.1-100 µg/ml
–	Etoposide	0.1-100 µg/ml
–	Fluoro-uracil	0.1-100 µg/ml
–	Girolline	0.36-360 µg/ml
–	Methotrexate	0.1-100 µg/ml
–	Novobiocin	0.1-100 µg/ml
–	Vinblastine	0.1-100 µg/ml
–	Vincristine	0.1-100 µg/ml

None of the compounds exhibited more than 5-10% inhibitory activity on the enzyme over the indicated range of concentration.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using not more than routine experimentation, nany equivalents to the specific embodiments of the tnvention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Cold Spring Harbor Laboratory

(ii) TITLE OF INVENTION: Novel Human *cdc25* Genes, Encoded Products and Uses Thereof

(iii) NUMBER OF SEQUENCES: 31

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: LAHIVE & COCKFIELD
(B) STREET: 60 State Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: U.S.A.
(F) ZIP: 02109

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII(text)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 24 April 1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Vincent, Matthew P.
(B) REGISTRATION NUMBER: 36,709
(C) REFERENCE/DOCKET NUMBER: MII-019-DV

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617-227-7400
(B) TELEFAX: 617-227-5941

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2419 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 460..2031

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGAAAGGCCG GCCTTGGCTG CGACAGCCTG GGTAAGAGGT GTAGGTCGGC TTGGTTTTCT 60

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GCTACCCGGA GCTGGGCAAG CGGGTTGGGA GAACAGCGAA GACAGCGTGA GCCTGGGCCG    120

TTGCCTCGAG GCTCTCGCCC GGCTTCTCTT GCCGACCCGC CACGTTTGTT TGGATTTAAT    180

CTTACAGCTG GTTGCCGGCG CCCGCCCGCC CGCTGGCCTC GCGGTGTGAG AGGGAAGCAC    240

CCGTGCCTGT GGCTGGTGGC TGGCGCCTGG AGGGTCCGCA CACCCGCCCG GCCGCGCCGC    300

TTTGCCCGCG GCAGCCGCGT CCCTGAACCG CGGAGTCGTG TTTGTGTTTG ACCCGCGGGC    360

GCCGGTGGCG CGCGGCCGAG GCCGGTGTCG GCGGGGCGGG GCGGTCGCGG CGGAGGCAGA    420

GGAAGAGGGA GCGGGAGCTC TGCGAGGCCG GGCGCCGCC ATG GAA CTG GGC CCG      474
                                          Met Glu Leu Gly Pro
                                           1               5

AGC CCC GCA CCG CGC CGC CTG CTC TTC GCC TGC AGC CCC CCT CCC GCG      522
Ser Pro Ala Pro Arg Arg Leu Leu Phe Ala Cys Ser Pro Pro Pro Ala
                 10                  15                  20

TCG CAG CCC GTC GTG AAG GCG CTA TTT GGC GCT TCA GCC GCC GGG GGA      570
Ser Gln Pro Val Val Lys Ala Leu Phe Gly Ala Ser Ala Ala Gly Gly
             25                  30                  35

CTG TCG CCT GTC ACC AAC CTG ACC GTC ACT ATG GAC CAG CTG CAG GGT      618
Leu Ser Pro Val Thr Asn Leu Thr Val Thr Met Asp Gln Leu Gln Gly
         40                  45                  50

CTG GGC AGT GAT TAT GAG CAA CCA CTG GAG GTG AAG AAC AAC AGT AAT      666
Leu Gly Ser Asp Tyr Glu Gln Pro Leu Glu Val Lys Asn Asn Ser Asn
     55                  60                  65

CTG CAG AGA ATG GGC TCC TCC GAG TCA ACA GAT TCA GGT TTC TGT CTA      714
Leu Gln Arg Met Gly Ser Ser Glu Ser Thr Asp Ser Gly Phe Cys Leu
 70                  75                  80                  85

GAT TCT CCT GGG CCA TTG GAC AGT AAA GAA AAC CTT GAA AAT CCT ATG      762
Asp Ser Pro Gly Pro Leu Asp Ser Lys Glu Asn Leu Glu Asn Pro Met
                 90                  95                 100

AGA AGA ATA CAT TCC CTA CCT CAA AAG CTG TTG GGA TGT AGT CCA GCT      810
Arg Arg Ile His Ser Leu Pro Gln Lys Leu Leu Gly Cys Ser Pro Ala
            105                 110                 115

CTG AAG AGG AGC CAT TCT GAT TCT CTT GAC CAT GAC ATC TTT CAG CTC      858
Leu Lys Arg Ser His Ser Asp Ser Leu Asp His Asp Ile Phe Gln Leu
        120                 125                 130

ATC GAC CCA GAT GAG AAC AAG GAA AAT GAA GCC TTT GAG TTT AAG AAG      906
Ile Asp Pro Asp Glu Asn Lys Glu Asn Glu Ala Phe Glu Phe Lys Lys
    135                 140                 145

CCA GTA AGA CCT GTA TCT CGT GGC TGC CTG CAC TCT CAT GGA CTC CAG      954
Pro Val Arg Pro Val Ser Arg Gly Cys Leu His Ser His Gly Leu Gln
150                 155                 160                 165

GAG GGT AAA GAT CTC TTC ACA CAG AGG CAG AAC TCT GCC CAG CTC GGA     1002
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Glu Gly Lys Asp Leu Phe Thr Gln Arg Gln Asn Ser Ala Gln Leu Gly
170 175 180

ATG CTT TCC TCA AAT GAA AGA GAT AGC AGT GAA CCA GGG AAT TTC ATT 1050
Met Leu Ser Ser Asn Glu Arg Asp Ser Ser Glu Pro Gly Asn Phe Ile
185 190 195

CCT CTT TTT ACA CCC CAG TCA CCT GTG ACA GCC ACT TTG TCT GAT GAG 1098
Pro Leu Phe Thr Pro Gln Ser Pro Val Thr Ala Thr Leu Ser Asp Glu
200 205 210

GAT GAT GGC TTC GTG GAC CTT CTC GAT GGA GAG AAT CTG AAG AAT GAG 1146
Asp Asp Gly Phe Val Asp Leu Leu Asp Gly Glu Asn Leu Lys Asn Glu
215 220 225

GAG GAG ACC CCC TCG TGC ATG GCA AGC CTC TGG ACA GCT CCT CTC GTC 1194
Glu Glu Thr Pro Ser Cys Met Ala Ser Leu Trp Thr Ala Pro Leu Val
230 235 240 245

ATG AGA ACT ACA AAC CTT GAC AAC CGA TGC AAG CTG TTT GAC TCC CCT 1242
Met Arg Thr Thr Asn Leu Asp Asn Arg Cys Lys Leu Phe Asp Ser Pro
250 255 260

TCC CTG TGT AGC TCC AGC ACT CGG TCA GTG TTG AAG AGA CCA GAA CGT 1290
Ser Leu Cys Ser Ser Ser Thr Arg Ser Val Leu Lys Arg Pro Glu Arg
265 270 275

TCT CAA GAG GAG TCT CCA CCT GGA AGT ACA AAG AGG AGG AAG AGC ATG 1338
Ser Gln Glu Glu Ser Pro Pro Gly Ser Thr Lys Arg Arg Lys Ser Met
280 285 290

TCT GGG GCC AGC CCC AAA GAG TCA ACT AAT CCA GAG AAG GCC CAT GAG 1386
Ser Gly Ala Ser Pro Lys Glu Ser Thr Asn Pro Glu Lys Ala His Glu
295 300 305

ACT CTT CAT CAG TCT TTA TCC CTG GCA TCT TCC CCC AAA GGA ACC ATT 1434
Thr Leu His Gln Ser Leu Ser Leu Ala Ser Ser Pro Lys Gly Thr Ile
310 315 320 325

GAG AAC ATT TTG GAC AAT GAC CCA AGG GAC CTT ATA GGA GAC TTC TCC 1482
Glu Asn Ile Leu Asp Asn Asp Pro Arg Asp Leu Ile Gly Asp Phe Ser
330 335 340

AAG GGT TAT CTC TTT CAT ACA GTT GCT GGG AAA CAT CAG GAT TTA AAA 1530
Lys Gly Tyr Leu Phe His Thr Val Ala Gly Lys His Gln Asp Leu Lys
345 350 355

TAC ATC TCT CCA GAA ATT ATG GCA TCT GTT TTG AAT GGC AAG TTT GCC 1578
Tyr Ile Ser Pro Glu Ile Met Ala Ser Val Leu Asn Gly Lys Phe Ala
360 365 370

AAC CTC ATT AAA GAG TTT GTT ATC ATC GAC TGT CGA TAC CCA TAT GAA 1626
Asn Leu Ile Lys Glu Phe Val Ile Ile Asp Cys Arg Tyr Pro Tyr Glu
375 380 385

TAC GAG GGA GGC CAC ATC AAG GGT GCA GTG AAC TTG CAC ATG GAA GAA 1674
Tyr Glu Gly Gly His Ile Lys Gly Ala Val Asn Leu His Met Glu Glu
390 395 400 405

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GAG GTT GAA GAC TTC TTA TTG AAG AAG CCC ATT GTA CCT ACT GAT GGC      1722
Glu Val Glu Asp Phe Leu Leu Lys Lys Pro Ile Val Pro Thr Asp Gly
                410                 415                 420

AAG CGT GTC ATT GTT GTG TTT CAC TGC GAG TTT TCT TCT GAG AGA GGT      1770
Lys Arg Val Ile Val Val Phe His Cys Glu Phe Ser Ser Glu Arg Gly
            425                 430                 435

CCC CGC ATG TGC CGG TAT GTG AGA GAG AGA GAT CGC CTG GGT AAT GAA      1818
Pro Arg Met Cys Arg Tyr Val Arg Glu Arg Asp Arg Leu Gly Asn Glu
        440                 445                 450

TAC CCC AAA CTC CAC TAC CCT GAG CTG TAT GTC CTG AAG GGG GGA TAC      1866
Tyr Pro Lys Leu His Tyr Pro Glu Leu Tyr Val Leu Lys Gly Gly Tyr
    455                 460                 465

AAG GAG TTC TTT ATG AAA TGC CAG TCT TAC TGT GAG CCC CCT AGC TAC      1914
Lys Glu Phe Phe Met Lys Cys Gln Ser Tyr Cys Glu Pro Pro Ser Tyr
470                 475                 480                 485

CGG CCC ATG CAC CAC GAG GAC TTT AAA GAA GAC CTG AAG AAG TTC CGC      1962
Arg Pro Met His His Glu Asp Phe Lys Glu Asp Leu Lys Lys Phe Arg
                490                 495                 500

ACC AAG AGC CGG ACC TGG GCA GGG GAG AAG AGC AAG AGG GAG ATG TAC      2010
Thr Lys Ser Arg Thr Trp Ala Gly Glu Lys Ser Lys Arg Glu Met Tyr
            505                 510                 515

AGT CGT CTG AAG AAG CTC TGAGGGCGGC AGGACCAGCC AGCAGCAGCC              2058
Ser Arg Leu Lys Lys Leu
        520

CAAGCTTCCC TCCATCCCCC TTTACCCTCT TTCCTGCAGA GAAACTTAAG CAAAGGGGAC    2118

AGCTGTGTGA CATTTGGAGA GGGGGCCTGG GACTTCCATG CCTTAAACCT ACCTCCCACA    2178

CTCCCAAGGT TGGAGCCCAG GGCATCTTGC TGGCTACGCC TCTTCTGTCC CTGTTAGACG    2238

TCCTCCGTCC ATATCAGAAC TGTGCCACAA TGCAGTTCTG AGCACCGTGT CAAGCTGCTC    2298

TGAGCCACAG TGGGATGAAC CAGCCGGGGC CTTATCGGGC TCCAGCATCT CATGAGGGGA    2358

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G                                                                    2419
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 523 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Glu Leu Gly Pro Ser Pro Ala Pro Arg Arg Leu Leu Phe Ala Cys
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Ser Pro Pro Pro Ala Ser Gln Pro Val Val Lys Ala Leu Phe Gly Ala
            20                  25                  30

Ser Ala Ala Gly Gly Leu Ser Pro Val Thr Asn Leu Thr Val Thr Met
        35                  40                  45

Asp Gln Leu Gln Gly Leu Gly Ser Asp Tyr Glu Gln Pro Leu Glu Val
    50                  55                  60

Lys Asn Asn Ser Asn Leu Gln Arg Met Gly Ser Ser Glu Ser Thr Asp
65                  70                  75                  80

Ser Gly Phe Cys Leu Asp Ser Pro Gly Pro Leu Asp Ser Lys Glu Asn
                85                  90                  95

Leu Glu Asn Pro Met Arg Arg Ile His Ser Leu Pro Gln Lys Leu Leu
            100                 105                 110

Gly Cys Ser Pro Ala Leu Lys Arg Ser His Ser Asp Ser Leu Asp His
        115                 120                 125

Asp Ile Phe Gln Leu Ile Asp Pro Asp Glu Asn Lys Glu Asn Glu Ala
    130                 135                 140

Phe Glu Phe Lys Lys Pro Val Arg Pro Val Ser Arg Gly Cys Leu His
145                 150                 155                 160

Ser His Gly Leu Gln Glu Gly Lys Asp Leu Phe Thr Gln Arg Gln Asn
                165                 170                 175

Ser Ala Gln Leu Gly Met Leu Ser Ser Asn Glu Arg Asp Ser Ser Glu
            180                 185                 190

Pro Gly Asn Phe Ile Pro Leu Phe Thr Pro Gln Ser Pro Val Thr Ala
        195                 200                 205

Thr Leu Ser Asp Glu Asp Asp Gly Phe Val Asp Leu Leu Asp Gly Glu
    210                 215                 220

Asn Leu Lys Asn Glu Glu Glu Thr Pro Ser Cys Met Ala Ser Leu Trp
225                 230                 235                 240

Thr Ala Pro Leu Val Met Arg Thr Thr Asn Leu Asp Asn Arg Cys Lys
                245                 250                 255

Leu Phe Asp Ser Pro Ser Leu Cys Ser Ser Ser Thr Arg Ser Val Leu
            260                 265                 270

Lys Arg Pro Glu Arg Ser Gln Glu Glu Ser Pro Pro Gly Ser Thr Lys
        275                 280                 285

Arg Arg Lys Ser Met Ser Gly Ala Ser Pro Lys Glu Ser Thr Asn Pro
    290                 295                 300
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Glu Lys Ala His Glu Thr Leu His Gln Ser Leu Ser Leu Ala Ser Ser
305 310 315 320

Pro Lys Gly Thr Ile Glu Asn Ile Leu Asp Asn Asp Pro Arg Asp Leu
325 330 335

Ile Gly Asp Phe Ser Lys Gly Tyr Leu Phe His Thr Val Ala Gly Lys
340 345 350

His Gln Asp Leu Lys Tyr Ile Ser Pro Glu Ile Met Ala Ser Val Leu
355 360 365

Asn Gly Lys Phe Ala Asn Leu Ile Lys Glu Phe Val Ile Ile Asp Cys
370 375 380

Arg Tyr Pro Tyr Glu Tyr Glu Gly Gly His Ile Lys Gly Ala Val Asn
385 390 395 400

Leu His Met Glu Glu Glu Val Glu Asp Phe Leu Leu Lys Lys Pro Ile
405 410 415

Val Pro Thr Asp Gly Lys Arg Val Ile Val Val Phe His Cys Glu Phe
420 425 430

Ser Ser Glu Arg Gly Pro Arg Met Cys Arg Tyr Val Arg Glu Arg Asp
435 440 445

Arg Leu Gly Asn Glu Tyr Pro Lys Leu His Tyr Pro Glu Leu Tyr Val
450 455 460

Leu Lys Gly Gly Tyr Lys Glu Phe Phe Met Lys Cys Gln Ser Tyr Cys
465 470 475 480

Glu Pro Pro Ser Tyr Arg Pro Met His His Glu Asp Phe Lys Glu Asp
485 490 495

Leu Lys Lys Phe Arg Thr Lys Ser Arg Thr Trp Ala Gly Glu Lys Ser
500 505 510

Lys Arg Glu Met Tyr Ser Arg Leu Lys Lys Leu
515 520

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2940 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 73..1773

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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CTGCCCTGCG CCCGGCCCTC CAGCCAGCCT GCCAGCTGTG CCGGCGTTTG TTGGTCTGCC      60

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              Met Glu Val Pro Gln Pro Glu Pro Ala Pro Gly Ser
                1               5                  10

GCT CTC AGT CCA GCA GGC GTG TGC GGT GGC GCC CAG CGT CCG GGC CAC       156
Ala Leu Ser Pro Ala Gly Val Cys Gly Gly Ala Gln Arg Pro Gly His
        15                  20                  25

CTC CCG GGC CTC CTG CTG GGA TCT CAT GGC CTC CTG GGG TCC CCG GTG       204
Leu Pro Gly Leu Leu Leu Gly Ser His Gly Leu Leu Gly Ser Pro Val
    30                  35                  40

CGG GCG GCC GCT TCC TCG CCG GTC ACC ACC CTC ACC CAG ACC ATG CAC       252
Arg Ala Ala Ala Ser Ser Pro Val Thr Thr Leu Thr Gln Thr Met His
45                  50                  55                  60

GAC CTC GCC GGG CTC GGC AGC CGC AGC CGC CTG ACG CAC CTA TCC CTG       300
Asp Leu Ala Gly Leu Gly Ser Arg Ser Arg Leu Thr His Leu Ser Leu
                65                  70                  75

TCT CGA CGG GCA TCC GAA TCC TCC CTG TCG TCT GAA TCC TCC GAA TCT       348
Ser Arg Arg Ala Ser Glu Ser Ser Leu Ser Ser Glu Ser Ser Glu Ser
            80                  85                  90

TCT GAT GCA GGT CTC TGC ATG GAT TCC CCC AGC CCT ATG GAC CCC CAC       396
Ser Asp Ala Gly Leu Cys Met Asp Ser Pro Ser Pro Met Asp Pro His
        95                  100                 105

ATG GCG GAG CAG ACG TTT GAA CAG GCC ATC CAG GCA GCC AGC CGG ATC       444
Met Ala Glu Gln Thr Phe Glu Gln Ala Ile Gln Ala Ala Ser Arg Ile
    110                 115                 120

ATT CGA AAC GAG CAG TTT GCC ATC AGA CGC TTC CAG TCT ATG CCG GTG       492
Ile Arg Asn Glu Gln Phe Ala Ile Arg Arg Phe Gln Ser Met Pro Val
125                 130                 135                 140

AGG CTG CTG GGC CAC AGC CCC GTG CTT CGG AAC ATC ACC AAC TCC CAG       540
Arg Leu Leu Gly His Ser Pro Val Leu Arg Asn Ile Thr Asn Ser Gln
                145                 150                 155

GCG CCC GAC GGC CGG AGG AAG AGC GAG GCG GGC AGT GGA GCT GCC AGC       588
Ala Pro Asp Gly Arg Arg Lys Ser Glu Ala Gly Ser Gly Ala Ala Ser
            160                 165                 170

AGC TCT GGG GAA GAC AAG GAG AAT GAT GGA TTT GTC TTC AAG ATG CCA       636
Ser Ser Gly Glu Asp Lys Glu Asn Asp Gly Phe Val Phe Lys Met Pro
        175                 180                 185

TGG AAG CCC ACA CAT CCC AGC TCC ACC CAT GCT CTG GCA GAG TGG GCC       684
Trp Lys Pro Thr His Pro Ser Ser Thr His Ala Leu Ala Glu Trp Ala
    190                 195                 200

AGC CGC AGG GAA GCC TTT GCC CAG AGA CCC AGC TCG GCC CCC GAC CTG       732
Ser Arg Arg Glu Ala Phe Ala Gln Arg Pro Ser Ser Ala Pro Asp Leu
205                 210                 215                 220
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ATG TGT CTC AGT CCT GAC CGG AAG ATG GAA GTG GAG GAG CTC AGC CCC 780
Met Cys Leu Ser Pro Asp Arg Lys Met Glu Val Glu Glu Leu Ser Pro
225 230 235

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Leu Ala Leu Gly Arg Phe Ser Leu Thr Pro Ala Glu Gly Asp Thr Glu
240 245 250

GAA GAT GAT GGA TTT GTG GAC ATC CTA GAG AGT GAC TTA AAG GAT GAT 876
Glu Asp Asp Gly Phe Val Asp Ile Leu Glu Ser Asp Leu Lys Asp Asp
255 260 265

GAT GCA GTT CCC CCA GGC ATG GAG AGT CTC ATT AGT GCC CCA CTG GTC 924
Asp Ala Val Pro Pro Gly Met Glu Ser Leu Ile Ser Ala Pro Leu Val
270 275 280

AAG ACC TTG GAA AAG GAA GAG GAA AAG GAC CTC GTC ATG TAC AGC AAG 972
Lys Thr Leu Glu Lys Glu Glu Glu Lys Asp Leu Val Met Tyr Ser Lys
285 290 295 300

TGC CAG CGG CTC TTC CGC TCT CCG TCC ATG CCC TGC AGC GTG ATC CGG 1020
Cys Gln Arg Leu Phe Arg Ser Pro Ser Met Pro Cys Ser Val Ile Arg
305 310 315

CCC ATC CTC AAG AGG CTG GAG CGG CCC CAG GAC AGG GAC ACG CCC GTG 1068
Pro Ile Leu Lys Arg Leu Glu Arg Pro Gln Asp Arg Asp Thr Pro Val
320 325 330

CAG AAT AAG CGG AGG CGG AGC GTG ACC CCT CCT GAG GAG CAG CAG GAG 1116
Gln Asn Lys Arg Arg Arg Ser Val Thr Pro Pro Glu Glu Gln Gln Glu
335 340 345

GCT GAG GAA CCT AAA GCC CGC GTC CTC CGC TCA AAA TCA CTG TGT CAC 1164
Ala Glu Glu Pro Lys Ala Arg Val Leu Arg Ser Lys Ser Leu Cys His
350 355 360

GAT GAG ATC GAG AAC CTC CTG GAC AGT GAC CAC CGA GAG CTG ATT GGA 1212
Asp Glu Ile Glu Asn Leu Leu Asp Ser Asp His Arg Glu Leu Ile Gly
365 370 375 380

GAT TAC TCT AAG GCC TTC CTC CTA CAG ACA GTA GAC GGA AAG CAC CAA 1260
Asp Tyr Ser Lys Ala Phe Leu Leu Gln Thr Val Asp Gly Lys His Gln
385 390 395

GAC CTC AAG TAC ATC TCA CCA GAA ACG ATG GTG GCC CTA TTG ACG GGC 1308
Asp Leu Lys Tyr Ile Ser Pro Glu Thr Met Val Ala Leu Leu Thr Gly
400 405 410

AAG TTC AGC AAC ATC GTG GAT AAG TTT GTG ATT GTA GAC TGC AGA TAC 1356
Lys Phe Ser Asn Ile Val Asp Lys Phe Val Ile Val Asp Cys Arg Tyr
415 420 425

CCC TAT GAA TAT GAA GGC GGG CAC ATC AAG ACT GCG GTG AAC TTG CCC 1404
Pro Tyr Glu Tyr Glu Gly Gly His Ile Lys Thr Ala Val Asn Leu Pro
430 435 440

CTG GAA CGC GAC GCC GAG AGC TTC CTA CTG AAG AGC CCC ATC GCG CCC 1452

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Leu Glu Arg Asp Ala Glu Ser Phe Leu Leu Lys Ser Pro Ile Ala Pro
445                 450                 455                 460

TGT AGC CTG GAC AAG AGA GTC ATC CTC ATT TTC CAC TGT GAA TTC TCA      1500
Cys Ser Leu Asp Lys Arg Val Ile Leu Ile Phe His Cys Glu Phe Ser
                465                 470                 475

TCT GAG CGT GGG CCC CGC ATG TGC CGT TTC ATC AGG GAA CGA GAC CGT      1548
Ser Glu Arg Gly Pro Arg Met Cys Arg Phe Ile Arg Glu Arg Asp Arg
            480                 485                 490

GCT GTC AAC GAC TAC CCC AGC CTC TAC TAC CCT GAG ATG TAT ATC CTG      1596
Ala Val Asn Asp Tyr Pro Ser Leu Tyr Tyr Pro Glu Met Tyr Ile Leu
        495                 500                 505

AAA GGC GGC TAC AAG GAG TTC TTC CCT CAG CAC CCG AAC TTC TGT GAA      1644
Lys Gly Gly Tyr Lys Glu Phe Phe Pro Gln His Pro Asn Phe Cys Glu
    510                 515                 520

CCC CAG GAC TAC CGG CCC ATG AAC CAC GAG GCC TTC AAG GAT GAG CTA      1692
Pro Gln Asp Tyr Arg Pro Met Asn His Glu Ala Phe Lys Asp Glu Leu
525                 530                 535                 540

AAG ACC TTC CGC CTC AAG ACT CGC AGC TGG GCT GGG GAG CGG AGC CGG      1740
Lys Thr Phe Arg Leu Lys Thr Arg Ser Trp Ala Gly Glu Arg Ser Arg
                545                 550                 555

CGG GAG CTC TGT AGC CGG CTG CAG GAC CAG TGAGGGGCCT GCGCCAGTCC        1790
Arg Glu Leu Cys Ser Arg Leu Gln Asp Gln
            560                 565

TGCTACCTCC CTTGCCTTTC GAGGCCTGAA GCCAGCTGCC CTATGGGCCT GCCGGGCTGA    1850

GGGCCTGCTG GAGGCCTCAG GTGCTGTCCA TGGGAAAGAT GGTGTGGTGT CCTGCCTGTC    1910

TGCCCCAGCC CAGATTCCCC TGTGTCATCC CATCATTTTC CATATCCTGG TGCCCCCCAC    1970

CCCTGGAAGA GCCCAGTCTG TTGAGTTAGT TAAGTTGGGT TAATACCAGC TTAAAGGCAG    2030

TATTTTGTGT CCTCCAGGAG CTTCTTGTTT CCTTGTTAGG GTTAACCCTT CATCTTCCTG    2090

TGTCCTGAAA CGCTCCTTTG TGTGTGTGTC AGCTGAGGCT GGGGAGAGCC GTGGTCCCTG    2150

AGGATGGGTC AGAGCTAAAC TCCTTCCTGG CCTGAGAGTC AGCTCTCTGC CCTGTGTACT    2210

TCCCGGGCCA GGGCTGCCCC TAATCTCTGT AGGAACCGTG GTATGTCTGC CATGTTGCCC    2270

CTTTCTCTTT TCCCCTTTCC TGTCCCACCA TACGAGCACC TCCAGCCTGA ACAGAAGCTC    2330

TTACTCTTTC CTATTTCAGT GTTACCTGTG TGCTTGGTCT GTTTGACTTT ACGCCCATCT    2390

CAGGACACTT CCGTAGACTG TTTAGGTTCC CCTGTCAAAT ATCAGTTACC CACTCGGTCC    2450

CAGTTTTGTT GCCCCAGAAA GGGATGTTAT TATCCTTGGG GGCTCCCAGG GCAAGGGTTA    2510

AGGCCTGAAT CATGAGCCTG CTGGAAGCCC AGCCCCTACT GCTGTGAACC CTGGGGCCTG    2570

ACTGCTCAGA ACTTGCTGCT GTCTTGTTGC GGATGGATGG AAGGTTGGAT GGATGGGTGG    2630
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ATGGCCGTGG ATGGCCGTGG ATGCGCAGTG CCTTGCATAC CCAAACCAGG TGGGAGCGTT      2690

TTGTTGAGCA TGACACCTGC AGCAGGAATA TATGTGTGCC TATTTGTGTG GACAAAAATA      2750

TTTACACTTA GGGTTTGGAG CTATTCAAGA GGAAATGTCA CAGAAGCAGC TAAACCAAGG      2810

ACTGAGCACC CTCTGGATTC TGAATCTCAA GATGGGGGCA GGGCTGTGCT TGAAGGCCCT      2870

GCTGAGTCAT CTGTTAGGGC CTTGGTTCAA TAAAGCACTG AGCAAGTTGA GAAAAAAAAA      2930

AAAAAAAAAA                                                             2940
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 566 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Glu Val Pro Gln Pro Glu Pro Ala Pro Gly Ser Ala Leu Ser Pro
 1               5                  10                  15

Ala Gly Val Cys Gly Gly Ala Gln Arg Pro Gly His Leu Pro Gly Leu
            20                  25                  30

Leu Leu Gly Ser His Gly Leu Leu Gly Ser Pro Val Arg Ala Ala Ala
        35                  40                  45

Ser Ser Pro Val Thr Thr Leu Thr Gln Thr Met His Asp Leu Ala Gly
    50                  55                  60

Leu Gly Ser Arg Ser Arg Leu Thr His Leu Ser Leu Ser Arg Arg Ala
65                  70                  75                  80

Ser Glu Ser Ser Leu Ser Ser Glu Ser Ser Glu Ser Ser Asp Ala Gly
                85                  90                  95

Leu Cys Met Asp Ser Pro Ser Pro Met Asp Pro His Met Ala Glu Gln
            100                 105                 110

Thr Phe Glu Gln Ala Ile Gln Ala Ala Ser Arg Ile Ile Arg Asn Glu
        115                 120                 125

Gln Phe Ala Ile Arg Arg Phe Gln Ser Met Pro Val Arg Leu Leu Gly
    130                 135                 140

His Ser Pro Val Leu Arg Asn Ile Thr Asn Ser Gln Ala Pro Asp Gly
145                 150                 155                 160

Arg Arg Lys Ser Glu Ala Gly Ser Gly Ala Ala Ser Ser Ser Gly Glu
                165                 170                 175
```

Asp Lys Glu Asn Asp Gly Phe Val Phe Lys Met Pro Trp Lys Pro Thr
180 185 190

His Pro Ser Ser Thr His Ala Leu Ala Glu Trp Ala Ser Arg Arg Glu
195 200 205

Ala Phe Ala Gln Arg Pro Ser Ser Ala Pro Asp Leu Met Cys Leu Ser
210 215 220

Pro Asp Arg Lys Met Glu Val Glu Glu Leu Ser Pro Leu Ala Leu Gly
225 230 235 240

Arg Phe Ser Leu Thr Pro Ala Glu Gly Asp Thr Glu Glu Asp Asp Gly
245 250 255

Phe Val Asp Ile Leu Glu Ser Asp Leu Lys Asp Asp Asp Ala Val Pro
260 265 270

Pro Gly Met Glu Ser Leu Ile Ser Ala Pro Leu Val Lys Thr Leu Glu
275 280 285

Lys Glu Glu Glu Lys Asp Leu Val Met Tyr Ser Lys Cys Gln Arg Leu
290 295 300

Phe Arg Ser Pro Ser Met Pro Cys Ser Val Ile Arg Pro Ile Leu Lys
305 310 315 320

Arg Leu Glu Arg Pro Gln Asp Arg Asp Thr Pro Val Gln Asn Lys Arg
325 330 335

Arg Arg Ser Val Thr Pro Pro Glu Glu Gln Gln Glu Ala Glu Glu Pro
340 345 350

Lys Ala Arg Val Leu Arg Ser Lys Ser Leu Cys His Asp Glu Ile Glu
355 360 365

Asn Leu Leu Asp Ser Asp His Arg Glu Leu Ile Gly Asp Tyr Ser Lys
370 375 380

Ala Phe Leu Leu Gln Thr Val Asp Gly Lys His Gln Asp Leu Lys Tyr
385 390 395 400

Ile Ser Pro Glu Thr Met Val Ala Leu Leu Thr Gly Lys Phe Ser Asn
405 410 415

Ile Val Asp Lys Phe Val Ile Val Asp Cys Arg Tyr Pro Tyr Glu Tyr
420 425 430

Glu Gly Gly His Ile Lys Thr Ala Val Asn Leu Pro Leu Glu Arg Asp
435 440 445

Ala Glu Ser Phe Leu Leu Lys Ser Pro Ile Ala Pro Cys Ser Leu Asp
450 455 460

Lys Arg Val Ile Leu Ile Phe His Cys Glu Phe Ser Ser Glu Arg Gly
465 470 475 480

Pro Arg Met Cys Arg Phe Ile Arg Glu Arg Asp Arg Ala Val Asn Asp

```
                485                 490                 495

Tyr Pro Ser Leu Tyr Tyr Pro Glu Met Tyr Ile Leu Lys Gly Gly Tyr
            500                 505                 510

Lys Glu Phe Phe Pro Gln His Pro Asn Phe Cys Glu Pro Gln Asp Tyr
        515                 520                 525

Arg Pro Met Asn His Glu Ala Phe Lys Asp Glu Leu Lys Thr Phe Arg
    530                 535                 540

Leu Lys Thr Arg Ser Trp Ala Gly Glu Arg Ser Arg Arg Glu Leu Cys
545                 550                 555                 560

Ser Arg Leu Gln Asp Gln
                565
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 205 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Leu Asp Asn Asp Pro Arg Asp Leu Ile Gly Asp Phe Ser Lys Gly Tyr
1               5                   10                  15

Leu Phe His Thr Val Ala Gly Lys His Gln Asp Leu Lys Tyr Ile Ser
            20                  25                  30

Pro Glu Ile Met Ala Ser Val Leu Asn Gly Lys Phe Ala Asn Leu Ile
        35                  40                  45

Lys Glu Phe Val Ile Ile Asp Cys Arg Tyr Pro Tyr Glu Tyr Glu Gly
    50                  55                  60

Gly His Ile Lys Gly Ala Val Asn Leu His Met Glu Glu Glu Val Glu
65                  70                  75                  80

Asp Phe Leu Leu Lys Lys Pro Ile Val Pro Xaa Xaa Xaa Xaa Xaa Xaa
                85                  90                  95

Xaa Xaa Thr Asp Gly Lys Arg Val Ile Val Val Phe His Cys Glu Phe
            100                 105                 110

Ser Ser Glu Arg Gly Pro Arg Met Cys Arg Tyr Val Arg Glu Arg Asp
        115                 120                 125

Arg Leu Gly Asn Glu Xaa Xaa Tyr Pro Lys Leu His Tyr Pro Glu Leu
    130                 135                 140

Tyr Val Leu Lys Gly Gly Tyr Lys Glu Phe Phe Met Lys Cys Gln Ser
145                 150                 155                 160
```

```
Tyr Cys Glu Pro Pro Ser Tyr Arg Pro Met His His Glu Asp Phe Lys
                165                 170                 175

Glu Asp Leu Lys Lys Phe Arg Thr Lys Ser Arg Thr Trp Ala Gly Glu
            180                 185                 190

Lys Ser Lys Arg Glu Met Tyr Ser Arg Leu Lys Lys Leu
        195                 200                 205
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 205 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Leu Asp Ser Asp His Arg Glu Leu Ile Gly Asp Tyr Ser Lys Ala Phe
1               5                   10                  15

Leu Leu Gln Thr Val Asp Gly Lys His Gln Asp Leu Lys Tyr Ile Ser
            20                  25                  30

Pro Glu Thr Val Met Ala Leu Leu Thr Gly Lys Phe Ser Asn Ile Val
        35                  40                  45

Asp Lys Phe Val Ile Val Asp Cys Arg Tyr Pro Tyr Glu Tyr Glu Gly
    50                  55                  60

Gly His Ile Lys Thr Ala Val Asn Leu Pro Leu Glu Arg Asp Ala Glu
65                  70                  75                  80

Ser Phe Leu Leu Lys Ser Pro Ile Ala Pro Cys Xaa Xaa Xaa Xaa Xaa
                85                  90                  95

Xaa Xaa Ser Leu Asp Lys Arg Val Ile Leu Ile Phe His Cys Glu Phe
            100                 105                 110

Ser Ser Glu Arg Gly Pro Arg Met Cys Arg Phe Ile Arg Glu Arg Asp
        115                 120                 125

Arg Ala Val Asn Asp Xaa Xaa Tyr Pro Ser Leu Tyr Tyr Pro Glu Met
    130                 135                 140

Tyr Ile Leu Lys Gly Gly Tyr Lys Glu Phe Phe Pro Gln His Pro Asn
145                 150                 155                 160

Phe Cys Glu Pro Gln Asp Tyr Arg Pro Met Asn His Glu Ala Phe Lys
                165                 170                 175

Asp Glu Leu Lys Thr Phe Arg Leu Lys Thr Arg Ser Trp Ala Gly Glu
            180                 185                 190
```

Arg Ser Arg Arg Glu Leu Cys Ser Arg Leu Gln Asp Gln
195 200 205

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 208 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Asp Ser Asn Gln Gly His Leu Ile Gly Asp Phe Ser Lys Val Cys
1 5 10 15

Ala Leu Pro Thr Val Ser Gly Lys His Gln Asp Leu Lys Tyr Val Asn
20 25 30

Pro Glu Thr Val Ala Ala Leu Leu Ser Gly Lys Phe Gln Gly Leu Ile
35 40 45

Glu Lys Phe Tyr Val Ile Asp Cys Arg Tyr Pro Tyr Glu Tyr Leu Gly
50 55 60

Gly His Ile Gln Gly Ala Leu Asn Leu Tyr Ser Gln Glu Glu Leu Phe
65 70 75 80

Asn Phe Phe Leu Lys Lys Pro Ile Val Pro Leu Xaa Xaa Xaa Xaa Xaa
85 90 95

Xaa Xaa Asp Thr Gln Lys Arg Ile Ile Ile Val Phe His Cys Glu Phe
100 105 110

Ser Ser Glu Arg Gly Pro Arg Met Cys Arg Cys Leu Arg Glu Glu Asp
115 120 125

Arg Ser Leu Asn Gln Xaa Xaa Tyr Pro Ala Leu Tyr Tyr Pro Glu Leu
130 135 140

Tyr Ile Leu Lys Gly Gly Tyr Arg Asp Phe Phe Pro Glu Tyr Met Glu
145 150 155 160

Leu Cys Glu Pro Gln Ser Tyr Cys Pro Met His His Gln Asp His Lys
165 170 175

Thr Glu Leu Leu Arg Cys Arg Ser Gln Ser Lys Val Gln Glu Gly Glu
180 185 190

Arg Gln Leu Arg Glu Gln Ile Ala Leu Leu Val Lys Asp Met Ser Pro
195 200 205

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 211 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Glu Asn Arg Asn Glu Pro Glu Leu Ile Gly Asp Phe Ser Lys Ala Tyr
1               5                   10                  15

Ser Leu Pro Leu Met Glu Gly Arg His Arg Asp Leu Lys Ser Ile Ser
            20                  25                  30

Ser Glu Thr Val Ala Arg Leu Leu Lys Gly Glu Phe Ser Asp Lys Val
        35                  40                  45

Ala Ser Tyr Arg Ile Ile Asp Cys Arg Tyr Pro Tyr Glu Phe Glu Gly
    50                  55                  60

Gly His Ile Glu Gly Ala Lys Asn Leu Tyr Thr Thr Glu Gln Ile Leu
65                  70                  75                  80

Asp Glu Phe Leu Thr Val Gln Gln Thr Glu Leu Gln Gln Gln Gln Asn
                85                  90                  95

Ala Glu Ser Gly His Lys Arg Asn Ile Ile Ile Phe His Cys Glu Phe
            100                 105                 110

Ser Ser Glu Arg Gly Pro Lys Met Ser Arg Gly Leu Arg Asn Leu Asp
        115                 120                 125

Arg Glu Arg Asn Thr Asn Ala Tyr Pro Ala Leu His Tyr Pro Glu Ile
    130                 135                 140

Tyr Leu Leu His Asn Gly Tyr Lys Glu Phe Phe Glu Ser His Val Glu
145                 150                 155                 160

Leu Cys Glu Pro His Ala Tyr Arg Thr Met Leu Asp Pro Ala Tyr Asn
                165                 170                 175

Glu Ala Tyr Arg His Phe Arg Ala Lys Ser Lys Ser Xaa Trp Asn Gly
            180                 185                 190

Asp Gly Leu Gly Gly Ala Thr Gly Arg Leu Lys Lys Ser Arg Ser Arg
        195                 200                 205

Leu Met Leu
    210
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 211 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Ser Thr Lys Glu Ser Glu Arg Phe Ile Ser Ser His Val Glu Asp Leu
1               5                   10                  15

Ser Leu Pro Cys Phe Ala Val Lys Glu Asp Ser Leu Lys Arg Ile Thr
            20                  25                  30

Gln Glu Thr Leu Leu Gly Leu Leu Asp Gly Lys Phe Lys Asp Ile Phe
        35                  40                  45

Asp Lys Cys Ile Ile Ile Asp Cys Arg Phe Glu Tyr Glu Tyr Leu Gly
    50                  55                  60

Gly His Ile Ser Thr Ala Val Asn Leu Asn Thr Lys Gln Ala Ile Val
65                  70                  75                  80

Asp Ala Phe Leu Ser Lys Pro Leu Thr Xaa Xaa Xaa Xaa Xaa Xaa Xaa
                85                  90                  95

Xaa Xaa Xaa Xaa His Val Arg Ala Xaa Leu Val Phe His Cys Glu His
            100                 105                 110

Ser Ala His Arg Ala Pro His Leu Ala Leu His Phe Arg Asn Thr Asp
        115                 120                 125

Arg Arg Met Asn Ser His Arg Tyr Pro Phe Leu Tyr Tyr Pro Glu Val
    130                 135                 140

Tyr Ile Leu His Gly Gly Tyr Lys Ser Phe Tyr Glu Asn His Lys Asn
145                 150                 155                 160

Arg Cys Asp Pro Ile Asn Tyr Val Pro Met Asn Asp Arg Ser His Val
                165                 170                 175

Asn Thr Cys Thr Lys Ala Met Asn Asn Phe Lys Arg Xaa Asn Ala Thr
            180                 185                 190

Phe Met Arg Thr Lys Ser Tyr Thr Phe Trp Pro Lys Cys Val Ser Phe
        195                 200                 205

Pro Arg Arg
    210
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Thr Asp Gly Lys Arg Val Ile Val Val Phe His Cys Glu Phe Ser Ser
1 5 10 15

Glu Arg Gly Pro Arg Met Cys Arg Tyr Val Arg Glu Arg Asp Arg Leu
20 25 30

Gly Asn Glu Xaa Xaa Tyr Pro Lys Leu His Tyr Pro Glu Leu Tyr Val
35 40 45

Leu Lys Gly Gly Tyr Lys Glu Phe Phe Met Lys Cys Gln Ser Tyr Cys
50 55 60

Glu Pro Pro Ser Tyr Arg Pro Met His His Glu
65 70 75

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ser Leu Asp Lys Arg Val Ile Leu Ile Phe His Cys Glu Phe Ser Ser
1 5 10 15

Glu Arg Gly Pro Arg Met Cys Arg Phe Ile Arg Glu Arg Asp Arg Ala
20 25 30

Val Asn Asp Xaa Xaa Tyr Pro Ser Leu Tyr Tyr Pro Glu Met Tyr Ile
35 40 45

Leu Lys Gly Gly Tyr Lys Glu Phe Phe Pro Gln His Pro Asn Phe Cys
50 55 60

Glu Pro Gln Asp Tyr Arg Pro Met Asn His Glu
65 70 75

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Asp Thr Gln Lys Arg Ile Ile Ile Val Phe His Cys Glu Phe Ser Ser
1 5 10 15

Glu Arg Gly Pro Arg Met Cys Arg Cys Leu Arg Glu Glu Asp Arg Ser
20 25 30

Leu Asn Gln Xaa Xaa Tyr Pro Ala Leu Tyr Tyr Pro Glu Leu Tyr Ile
35 40 45

Leu Lys Gly Gly Tyr Arg Asp Phe Phe Pro Glu Tyr Met Glu Leu Cys
50 55 60

Glu Pro Gln Ser Tyr Cys Pro Met His His Gln
65 70 75

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ser Gly His Lys Arg Asn Ile Ile Ile Phe His Cys Glu Phe Ser Ser
1 5 10 15

Glu Arg Gly Pro Lys Met Ser Arg Gly Leu Arg Asn Leu Asp Arg Glu
20 25 30

Arg Asn Thr Asn Ala Tyr Pro Ala Leu His Tyr Pro Glu Ile Tyr Leu
35 40 45

Leu His Asn Gly Tyr Lys Glu Phe Phe Glu Ser His Val Glu Leu Cys
50 55 60

Glu Pro His Ala Tyr Arg Thr Met Leu Asp Pro
65 70 75

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Xaa Xaa His Val Arg Ala Xaa Leu Val Phe His Cys Glu His Ser Ala
1 5 10 15

His Arg Ala Pro His Leu Ala Leu His Phe Arg Asn Thr Asp Arg Arg
20 25 30

Met Asn Ser His Arg Tyr Pro Phe Leu Tyr Tyr Pro Glu Val Tyr Ile
35 40 45

Leu His Gly Gly Tyr Lys Ser Phe Tyr Glu Asn His Lys Asn Arg Cys
50 55 60

Asp Pro Ile Asn Tyr Val Pro Met Asn Asp Arg
65 70 75

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Xaa Xaa Xaa Xaa Asn Glu Pro Val Leu Val His Cys Ala Ala Gly Val
1 5 10 15

Asn Arg Ser Gly Ala Met Ile Leu Ala Xaa Xaa Xaa Xaa Tyr Leu Met
20 25 30

Ser Lys Asn Lys Glu Ser Leu Pro Met Leu Tyr Phe Leu Tyr Val Tyr
35 40 45

His Ser Met Arg Asp Leu Arg Xaa Gly Ala Phe Val Glu Asn Pro Ser
50 55 60

Phe Lys Arg Xaa Xaa Xaa Xaa Gln Ile Ile Glu
65 70 75

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Leu Ser Pro Glu Asn Gly Pro Ile Val Val His Cys Ser Ala Gly Ile
1 5 10 15

Gly Arg Ser Gly Thr Phe Cys Leu Ala Asp Thr Cys Leu Leu Leu Met
20 25 30

Asp Lys Arg Lys Asp Pro Ser Ser Val Asp Xaa Ile Lys Lys Val Leu
35 40 45

Leu Glu Met Arg Arg Phe Arg Met Gly Xaa Leu Ile Gln Thr Ala Asp
50 55 60

Gln Leu Arg Phe Ser Tyr Leu Ala Val Ile Glu
65 70 75

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Leu Ser Pro Glu His Gly Pro Val Val Val His Cys Ser Ala Gly Ile
1               5                   10                  15

Gly Arg Ser Gly Thr Phe Cys Leu Ala Asp Thr Cys Leu Leu Leu Met
            20                  25                  30

Asp Lys Arg Lys Asp Pro Ser Ser Val Asp Xaa Leu Lys Lys Val Leu
        35                  40                  45

Leu Glu Met Arg Lys Phe Arg Met Gly Xaa Leu Ile Gln Thr Ala Asp
    50                  55                  60

Gln Leu Arg Phe Ser Tyr Leu Ala Val Ile Glu
65                  70                  75
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Leu Asn Pro Asp His Gly Pro Ala Val Ile His Cys Ser Ala Gly Ile
1               5                   10                  15

Gly Arg Ser Gly Thr Phe Ser Leu Val Asp Thr Cys Leu Val Leu Met
            20                  25                  30

Glu Lys Gly Asp Asp Ile Asn Xaa Xaa Xaa Xaa Ile Lys Gln Val Leu
        35                  40                  45

Leu Asn Met Arg Lys Tyr Arg Met Gly Xaa Leu Ile Gln Thr Pro Asp
    50                  55                  60

Gln Leu Arg Phe Ser Tyr Met Ala Ile Ile Glu
65                  70                  75
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
Leu Ala Val Asn Asp Val Asp Ala Glu Asp Gly Ala Asp Pro Asn Leu
1               5                   10                  15

Cys Ser Glu Tyr Val Lys Asp Ile Tyr Ala Tyr Leu Arg Gln Leu Glu
            20                  25                  30

Glu Glu Gln Ala Val Arg Pro Lys Tyr Leu Leu Gly Arg Glu Val Thr
        35                  40                  45

Gly Asn Met Arg Ala Ile Leu Ile Asp Trp Leu Val Gln Xaa Xaa Val
    50                  55                  60

Gln Met Lys Phe Arg Leu Leu Gln Xaa Xaa Glu
65                  70                  75
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Ile His Val Lys Asp Val Asp Ala Asp Asp Asp Gly Asn Pro Met Leu
1               5                   10                  15

Cys Ser Glu Tyr Val Lys Asp Ile Tyr Ala Tyr Leu Arg Ser Leu Glu
            20                  25                  30

Asp Ala Gln Ala Val Arg Gln Asn Tyr Leu His Gly Gln Glu Val Thr
        35                  40                  45

Gly Asn Met Arg Ala Ile Leu Ile Asp Trp Leu Val Gln Xaa Xaa Val
    50                  55                  60

Gln Met Arg Phe Arg Leu Leu Gln Xaa Xaa Glu
65                  70                  75
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Thr Ser Val Glu Asp Ile Asp Ala Asp Asp Gly Gly Asn Pro Gln Leu
1 5 10 15

Cys Ser Asp Tyr Val Met Asp Ile Tyr Asn Tyr Leu Lys Gln Leu Glu
20 25 30

Val Gln Gln Ser Val His Pro Cys Tyr Leu Glu Gly Lys Glu Ile Asn
35 40 45

Glu Arg Met Arg Ala Ile Leu Val Asp Trp Leu Val Gln Xaa Xaa Val
50 55 60

His Ser Arg Phe Gln Leu Leu Gln Xaa Xaa Glu
65 70 75

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Leu Cys Cys Glu Val Glu Thr Ile Arg Arg Ala Tyr Pro Asp Ala Asn
1 5 10 15

Leu Leu Asn Asp Arg Val Leu Arg Ala Met Leu Lys Ala Glu Glu Thr
20 25 30

Cys Ala Pro Ser Val Ser Tyr Phe Lys Cys Val Gln Lys Glu Val Leu
35 40 45

Pro Ser Met Arg Lys Ile Val Ala Thr Trp Met Leu Glu Xaa Xaa Val
50 55 60

Cys Glu Glu Gln Lys Cys Glu Glu Xaa Xaa Glu
65 70 75

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Ser Ile Val Leu Glu Asp Glu Lys Pro Val Ser Val Asn Glu Val

1 5 10 15

Pro Asp Tyr His Glu Asp Ile His Thr Tyr Leu Arg Glu Met Glu Val
20 25 30

Lys Cys Lys Pro Lys Val Gly Tyr Met Lys Lys Gln Pro Asp Ile Thr
35 40 45

Asn Ser Met Arg Ala Ile Leu Val Asp Trp Leu Val Glu Xaa Xaa Val
50 55 60

Gly Glu Glu Tyr Lys Leu Gln Asn Xaa Xaa Glu
65 70 75

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Ile Ile Asp Cys Arg Thr Phe Pro Glu Tyr Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Ala Thr Ile Ala Thr Ile Gly Ala Thr Thr Gly Cys Cys Gly Ile Thr
1 5 10 15

Ala Thr Cys Cys Cys Ile Thr Ala Cys Thr Gly Ala
20 25

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Ala Thr Ile Ala Thr Ile Gly Ala Thr Thr Gly Cys Cys Gly Ile Thr
1 5 10 15

Ala Thr Cys Gly Ala Ile Thr Ala Cys Thr Gly Ala
20 25

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATAGAACTTC AGCAAGTGAG AAAGTA 26

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Cys Gln Gly Ala Leu Asn Leu Tyr Ser Gln Glu Glu Leu Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Cys Lys Gly Ala Val Asn Leu His Met Glu Glu Glu Val Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Cys Lys Lys Lys Val Glu Lys Ile Gly Glu Gly Thr Tyr Gly Val Val
1 5 10 15

Tyr Lys

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Leu Val Phe His Cys Glu Xaa Xaa Xaa Xaa Arg
1 5 10

Claims

1. Purified *CDC25*A protein which is of mammalian origin.

2. The *CDC25*A protein of claim 1, which protein is a product of recombinant expression.

3. Purified *CDC25*B protein which is of mammalian origin.

4. The *CDC25*B protein of claim 3, which protein is a product of recombinant expression.

5. A recombinant *CDC25*A polypeptide comprising an amino acid sequence designated in SEQ ID NO: 2, which recombinant polypeptide possesses an endogenous tyrosine phosphatase activity.

6. The recombinant polypeptide of Claim 5, which recombinant polypeptide is a fusion protein.

7. The recombinant polypeptide of Claim 6, wherein the fusion protein further includes a glutathione-S-transferase amino acid sequence.

8. The recombinant polypeptide of Claim 5, which endogenous tyrosine phosphatase activity hydrolyzes p-nitrophenylphosphate.

9. The recombinant polypeptide of Claim 5, which recombinant polypeptide rescues a mutant *cdc25-22* strain of fission yeast.

10. The recombinant polypeptide of Claim 5, which endogenous tyrosine phosphatase activity dephosphorylates a phosphorylated catalytic subunit of an M-phase kinase.

11. The recombinant polypeptide of Claim 5, which endogenous tyrosine phosphatase activity dephosphorylates a phosphorylated CDC2 kinase.

12. A recombinant *CDC25*B polypeptide comprising an amino acid sequence designated in SEQ ID NO: 4, which recombinant polypeptide possesses an endogenous tyrosine phosphatase activity.

13. The recombinant polypeptide of Claim 12, which recombinant polypeptide is a fusion protein.

14. The recombinant polypeptide of Claim 13, wherein the fusion protein further includes a glutathione-S-transferase amino acid sequence.

15. The recombinant polypeptide of Claim 12, which recombinant polypeptide hydrolyzes p-nitrophenylphosphate.

16. The recombinant polypeptide of Claim 12, which recombinant polypeptide rescues a mutant *cdc25-22* strain of fission yeast.

17. The recombinant polypeptide of Claim 12, which endogenous tyrosine phosphatase activity dephosphorylates a phosphorylated catalytic subunit of an M-phase kinase.

18. The recombinant polypeptide of Claim 12, which endogenous tyrosine phosphatase activity dephosphorylates a phosphorylated CDC2 kinase.

19. A recombinant *CDC25*A polypeptide encoded by a nucleic acid comprising a nucleotide sequence which

(i) specifically hybridizes under high stringency conditions to the *CDC25A* gene designated by SEQ ID No. 1, and

(ii) encodes a polypeptide which possesses an endogenous catalytic phosphatase activity.

20. The recombinant polypeptide of Claim 19, which recombinant polypeptide is a fusion protein.

21. A recombinant *CDC25*B polypeptide encoded by a nucleic acid comprising a nucleotide sequence which

(i) specifically hybridizes under high stringency conditions to the *CDC25B* gene designated by SEQ ID No. 3, and

(ii) encodes a polypeptide which possesses an endogenous catalytic phosphatase activity.

22. The recombinant polypeptide of Claim 21, which recombinant polypeptide is a fusion protein.

23. A recombinant *CDC25*A polypeptide comprising an amino acid sequence designated in SEQ ID NO: 2, which recombinant polypeptide binds to a phosphotyrosine containing cyclin dependent kinase.

24. The recombinant polypeptide of Claim 23, which cyclin dependent kinase is a CDC2 kinase.

25. A recombinant *CDC25B* polypeptide comprising an amino acid sequence designated in SEQ ID NO: 4, which recombinant polypeptide binds to a phosphotyrosine containing cyclin dependent kinase.

26. The recombinant polypeptide of Claim 25, which cyclin dependent kinase is a CDC2 kinase.

27. A purified or recombinant polypeptide which is immunoprecipitated by an antibody against the *CDC25A* protein designated by SEQ ID NO: 2, which polypeptide binds to a phosphotyrosine containing cyclin dependent kinase.

28. A purified or recombinant polypeptide which is immunoprecipitated by an antibody against the *CDC25A* protein designated by SEQ ID NO: 4, which polypeptide binds to a phosphotyrosine containing cyclin dependent kinase.

29. An antibody which specifically binds a mammalian *CDC25*A protein.

30. An antibody which specifically binds a mammalian *CDC25*B protein.

NOVEL HUMAN cdc25 GENES, ENCODED PRODUCTS AND USES THEREFOR

Abstract of the Disclosure

Two previously undescribed human cdc25 genes, designated cdc25 A and cdc25 B, which have been shown to have an endogenous tyrosine phosphatase activity that can be specifically activated by B-type cyclin, in the complete absence of cdc2 are described. As a result of this work, new approaches to regulating the cell cycle in eukaryotic cells and, particularly, to regulating the activity of tyrosine specific phosphatases which play a key role in the cell cycle are available. Applicant's invention relates to methods of regulating the cell cycle and, specifically, to regulating activation of cdc2-kinase, through alteration of the activity and/or levels of tyrosine phosphatases or through alteration of the interaction of components of MPF. The present invention also relates to agents or compositions useful in the method of regulating (inhibiting or enhancing) the cell cycle. Such agents or compositions can be inhibitors (such as low molecular weight peptides or compounds, either organic or inorganic) of the catalytic activity of tyrosine specific PTPases (particularly cdc25), blocking agents which interfere with interaction or binding of the tyrosine specific PTPase with cyclin or the cyclin/cdc2 complex, or agents which interfere directly with the catalytic activity of the PTPases. The invention also pertains to an assay for identifying agents which after stimulation of kinase activity of pre-MPF and thus alter activation of MPF and entry into mitosis. Such agents are also the subject of this invention.

```
CGAAAGGCCG GCCTTGGCTG CGACAGCCTG GGTAAGAGGT GTAGGTCGGC TTGGTTTTCT       60

GCTACCCGGA GCTGGGCAAG CGGGTTGGGA GAACAGCGAA GACAGCGTGA GCCTGGGCCG      120

TTGCCTCGAG GCTCTCGCCC GGCTTCTCTT GCCGACCCGC CACGTTTGTT TGGATTTAAT      180

CTTACAGCTG GTTGCCGGCG CCCGCCCGCC CGCTGGCCTC GCGGTGTGAG AGGGAAGCAC      240

CCGTGCCTGT GGCTGGTGGC TGGCGCCTGG AGGGTCCGCA CACCCGCCCG GCCGCGCCGC      300

TTTGCCCGCG GCAGCCGCGT CCCTGAACCG CGGAGTCGTG TTTGTGTTTG ACCCGCGGGC      360

GCCGGTGGCG CGCGGCCGAG GCCGGTGTCG GCGGGGCGGG GCGGTCGCGG CGGAGGCAGA      420

GGAAGAGGGA GCGGGAGCTC TGCGAGGCCG GGCGCCGCC ATG GAA CTG GGC CCG      474
                                          Met Glu Leu Gly Pro
                                           1               5

AGC CCC GCA CCG CGC CGC CTG CTC TTC GCC TGC AGC CCC CCT CCC GCG      522
Ser Pro Ala Pro Arg Arg Leu Leu Phe Ala Cys Ser Pro Pro Pro Ala
                10                  15                  20

TCG CAG CCC GTC GTG AAG GCG CTA TTT GGC GCT TCA GCC GCC GGG GGA      570
Ser Gln Pro Val Val Lys Ala Leu Phe Gly Ala Ser Ala Ala Gly Gly
            25                  30                  35

CTG TCG CCT GTC ACC AAC CTG ACC GTC ACT ATG GAC CAG CTG CAG GGT      618
Leu Ser Pro Val Thr Asn Leu Thr Val Thr Met Asp Gln Leu Gln Gly
        40                  45                  50

CTG GGC AGT GAT TAT GAG CAA CCA CTG GAG GTG AAG AAC AAC AGT AAT      666
Leu Gly Ser Asp Tyr Glu Gln Pro Leu Glu Val Lys Asn Asn Ser Asn
    55                  60                  65

CTG CAG AGA ATG GGC TCC TCC GAG TCA ACA GAT TCA GGT TTC TGT CTA      714
Leu Gln Arg Met Gly Ser Ser Glu Ser Thr Asp Ser Gly Phe Cys Leu
70                  75                  80                  85

GAT TCT CCT GGG CCA TTG GAC AGT AAA GAA AAC CTT GAA AAT CCT ATG      762
Asp Ser Pro Gly Pro Leu Asp Ser Lys Glu Asn Leu Glu Asn Pro Met
                90                  95                  100

AGA AGA ATA CAT TCC CTA CCT CAA AAG CTG TTG GGA TGT AGT CCA GCT      810
Arg Arg Ile His Ser Leu Pro Gln Lys Leu Leu Gly Cys Ser Pro Ala
            105                 110                 115

CTG AAG AGG AGC CAT TCT GAT TCT CTT GAC CAT GAC ATC TTT CAG CTC      858
Leu Lys Arg Ser His Ser Asp Ser Leu Asp His Asp Ile Phe Gln Leu
        120                 125                 130

ATC GAC CCA GAT GAG AAC AAG GAA AAT GAA GCC TTT GAG TTT AAG AAG      906
Ile Asp Pro Asp Glu Asn Lys Glu Asn Glu Ala Phe Glu Phe Lys Lys
    135                 140                 145

CCA GTA AGA CCT GTA TCT CGT GGC TGC CTG CAC TCT CAT GGA CTC CAG      954
Pro Val Arg Pro Val Ser Arg Gly Cys Leu His Ser His Gly Leu Gln
150                 155                 160                 165

GAG GGT AAA GAT CTC TTC ACA CAG AGG CAG AAC TCT GCC CAG CTC GGA     1002
Glu Gly Lys Asp Leu Phe Thr Gln Arg Gln Asn Ser Ala Gln Leu Gly
                170                 175                 180
```

Figure 1(a) - Panel A

```
ATG CTT TCC TCA AAT GAA AGA GAT AGC AGT GAA CCA GGG AAT TTC ATT      1050
Met Leu Ser Ser Asn Glu Arg Asp Ser Ser Glu Pro Gly Asn Phe Ile
            185                 190                 195

CCT CTT TTT ACA CCC CAG TCA CCT GTG ACA GCC ACT TTG TCT GAT GAG      1098
Pro Leu Phe Thr Pro Gln Ser Pro Val Thr Ala Thr Leu Ser Asp Glu
        200                 205                 210

GAT GAT GGC TTC GTG GAC CTT CTC GAT GGA GAG AAT CTG AAG AAT GAG      1146
Asp Asp Gly Phe Val Asp Leu Leu Asp Gly Glu Asn Leu Lys Asn Glu
    215                 220                 225

GAG GAG ACC CCC TCG TGC ATG GCA AGC CTC TGG ACA GCT CCT CTC GTC      1194
Glu Glu Thr Pro Ser Cys Met Ala Ser Leu Trp Thr Ala Pro Leu Val
230                 235                 240                 245

ATG AGA ACT ACA AAC CTT GAC AAC CGA TGC AAG CTG TTT GAC TCC CCT      1242
Met Arg Thr Thr Asn Leu Asp Asn Arg Cys Lys Leu Phe Asp Ser Pro
                250                 255                 260

TCC CTG TGT AGC TCC AGC ACT CGG TCA GTG TTG AAG AGA CCA GAA CGT      1290
Ser Leu Cys Ser Ser Ser Thr Arg Ser Val Leu Lys Arg Pro Glu Arg
            265                 270                 275

TCT CAA GAG GAG TCT CCA CCT GGA AGT ACA AAG AGG AGG AAG AGC ATG      1338
Ser Gln Glu Glu Ser Pro Pro Gly Ser Thr Lys Arg Arg Lys Ser Met
        280                 285                 290

TCT GGG GCC AGC CCC AAA GAG TCA ACT AAT CCA GAG AAG GCC CAT GAG      1386
Ser Gly Ala Ser Pro Lys Glu Ser Thr Asn Pro Glu Lys Ala His Glu
    295                 300                 305

ACT CTT CAT CAG TCT TTA TCC CTG GCA TCT TCC CCC AAA GGA ACC ATT      1434
Thr Leu His Gln Ser Leu Ser Leu Ala Ser Ser Pro Lys Gly Thr Ile
310                 315                 320                 325

GAG AAC ATT TTG GAC AAT GAC CCA AGG GAC CTT ATA GGA GAC TTC TCC      1482
Glu Asn Ile Leu Asp Asn Asp Pro Arg Asp Leu Ile Gly Asp Phe Ser
                330                 335                 340

AAG GGT TAT CTC TTT CAT ACA GTT GCT GGG AAA CAT CAG GAT TTA AAA      1530
Lys Gly Tyr Leu Phe His Thr Val Ala Gly Lys His Gln Asp Leu Lys
            345                 350                 355

TAC ATC TCT CCA GAA ATT ATG GCA TCT GTT TTG AAT GGC AAG TTT GCC      1578
Tyr Ile Ser Pro Glu Ile Met Ala Ser Val Leu Asn Gly Lys Phe Ala
        360                 365                 370

AAC CTC ATT AAA GAG TTT GTT ATC ATC GAC TGT CGA TAC CCA TAT GAA      1626
Asn Leu Ile Lys Glu Phe Val Ile Ile Asp Cys Arg Tyr Pro Tyr Glu
    375                 380                 385

TAC GAG GGA GGC CAC ATC AAG GGT GCA GTG AAC TTG CAC ATG GAA GAA      1674
Tyr Glu Gly Gly His Ile Lys Gly Ala Val Asn Leu His Met Glu Glu
390                 395                 400                 405

GAG GTT GAA GAC TTC TTA TTG AAG AAG CCC ATT GTA CCT ACT GAT GGC      1722
Glu Val Glu Asp Phe Leu Leu Lys Lys Pro Ile Val Pro Thr Asp Gly
                410                 415                 420
```

Figure 1(b) - Panel A

```
AAG CGT GTC ATT GTT GTG TTT CAC TGC GAG TTT TCT TCT GAG AGA GGT        1770
Lys Arg Val Ile Val Val Phe His Cys Glu Phe Ser Ser Glu Arg Gly
            425                 430                 435

CCC CGC ATG TGC CGG TAT GTG AGA GAG AGA GAT CGC CTG GGT AAT GAA        1818
Pro Arg Met Cys Arg Tyr Val Arg Glu Arg Asp Arg Leu Gly Asn Glu
        440                 445                 450

TAC CCC AAA CTC CAC TAC CCT GAG CTG TAT GTC CTG AAG GGG GGA TAC        1866
Tyr Pro Lys Leu His Tyr Pro Glu Leu Tyr Val Leu Lys Gly Gly Tyr
    455                 460                 465

AAG GAG TTC TTT ATG AAA TGC CAG TCT TAC TGT GAG CCC CCT AGC TAC        1914
Lys Glu Phe Phe Met Lys Cys Gln Ser Tyr Cys Glu Pro Pro Ser Tyr
470                 475                 480                 485

CGG CCC ATG CAC CAC GAG GAC TTT AAA GAA GAC CTG AAG AAG TTC CGC        1962
Arg Pro Met His His Glu Asp Phe Lys Glu Asp Leu Lys Lys Phe Arg
                490                 495                 500

ACC AAG AGC CGG ACC TGG GCA GGG GAG AAG AGC AAG AGG GAG ATG TAC        2010
Thr Lys Ser Arg Thr Trp Ala Gly Glu Lys Ser Lys Arg Glu Met Tyr
            505                 510                 515

AGT CGT CTG AAG AAG CTC TGAGGGCGGC ACGACCAGCC AGCAGCAGCC               2058
Ser Arg Leu Lys Lys Leu
        520

CAAGCTTCCC TCCATCCCCC TTTACCCTCT TTCCTGCAGA GAAACTTAAG CAAAGGGGAC      2118

AGCTGTGTGA CATTTGGAGA GGGGGCCTGG GACTTCCATG CCTTAAACCT ACCTCCCACA      2178

CTCCCAAGGT TGGAGCCCAG GGCATCTTGC TGGCTACGCC TCTTCTGTCC CTGTTAGACG      2238

TCCTCCGTCC ATATCAGAAC TGTGCCACAA TGCAGTTCTG AGCACCGTGT CAAGCTGCTC      2298

TCAGCCACAG TGGGATGAAC CAGCCGGGGC CTTATCGGGC TCCAGCATCT CATGAGGGGA      2358

GAGGAGACGG AGGGGAGTAG AGAAGTTTAC ACAGAAATGC TGCTGGCCAA ATAGCAAAGA      2418

G                                                                      2419
```

Figure 1(c) - Panel A

CTGCCCTGCG CCCGGCCCTC CAGCCAGCCT GCCAGCTGTG CCGGCGTTTG TTGGTCTGCC 60

GGCCCCGCCG CG ATG GAG GTG CCC CAG CCG GAG CCC GCG CCA GGC TCG 108
Met Glu Val Pro Gln Pro Glu Pro Ala Pro Gly Ser
1 5 10

GCT CTC AGT CCA GCA GGC GTG TGC GGT GGC GCC CAG CGT CCG GGC CAC 156
Ala Leu Ser Pro Ala Gly Val Cys Gly Gly Ala Gln Arg Pro Gly His
15 20 25

CTC CCG GGC CTC CTG CTG GGA TCT CAT GGC CTC CTG GGG TCC CCG GTG 204
Leu Pro Gly Leu Leu Leu Gly Ser His Gly Leu Leu Gly Ser Pro Val
30 35 40

CGG GCG GCC GCT TCC TCG CCG GTC ACC ACC CTC ACC CAG ACC ATG CAC 252
Arg Ala Ala Ala Ser Ser Pro Val Thr Thr Leu Thr Gln Thr Met His
45 50 55 60

GAC CTC GCC GGG CTC GGC AGC CGC AGC CGC CTG ACG CAC CTA TCC CTG 300
Asp Leu Ala Gly Leu Gly Ser Arg Ser Arg Leu Thr His Leu Ser Leu
65 70 75

TCT CGA CGG GCA TCC GAA TCC TCC CTG TCG TCT GAA TCC TCC GAA TCT 348
Ser Arg Arg Ala Ser Glu Ser Ser Leu Ser Ser Glu Ser Ser Glu Ser
80 85 90

TCT GAT GCA GGT CTC TGC ATG GAT TCC CCC AGC CCT ATG GAC CCC CAC 396
Ser Asp Ala Gly Leu Cys Met Asp Ser Pro Ser Pro Met Asp Pro His
95 100 105

ATG GCG GAG CAG ACG TTT GAA CAG GCC ATC CAG GCA GCC AGC CGG ATC 444
Met Ala Glu Gln Thr Phe Glu Gln Ala Ile Gln Ala Ala Ser Arg Ile
110 115 120

ATT CGA AAC GAG CAG TTT GCC ATC AGA CGC TTC CAG TCT ATG CCG GTG 492
Ile Arg Asn Glu Gln Phe Ala Ile Arg Arg Phe Gln Ser Met Pro Val
125 130 135 140

AGG CTG CTG GGC CAC AGC CCC GTG CTT CGG AAC ATC ACC AAC TCC CAG 540
Arg Leu Leu Gly His Ser Pro Val Leu Arg Asn Ile Thr Asn Ser Gln
145 150 155

GCG CCC GAC GGC CGG AGG AAG AGC GAG GCG GGC AGT GGA GCT GCC AGC 588
Ala Pro Asp Gly Arg Arg Lys Ser Glu Ala Gly Ser Gly Ala Ala Ser
160 165 170

AGC TCT GGG GAA GAC AAG GAG AAT GAT GGA TTT GTC TTC AAG ATG CCA 636
Ser Ser Gly Glu Asp Lys Glu Asn Asp Gly Phe Val Phe Lys Met Pro
175 180 185

TGG AAG CCC ACA CAT CCC AGC TCC ACC CAT GCT CTG GCA GAG TGG GCC 684
Trp Lys Pro Thr His Pro Ser Ser Thr His Ala Leu Ala Glu Trp Ala
190 195 200

AGC CGC AGG GAA GCC TTT GCC CAG AGA CCC AGC TCG GCC CCC GAC CTG 732
Ser Arg Arg Glu Ala Phe Ala Gln Arg Pro Ser Ser Ala Pro Asp Leu
205 210 215 220

ATG TGT CTC AGT CCT GAC CGG AAG ATG GAA GTG GAG GAG CTC AGC CCC 780
Met Cys Leu Ser Pro Asp Arg Lys Met Glu Val Glu Glu Leu Ser Pro
225 230 235

Figure 1(d) - Panel B

```
CTG GCC CTA GGT CGC TTC TCT CTG ACC CCT GCA GAG GGG GAT ACT GAG        828
Leu Ala Leu Gly Arg Phe Ser Leu Thr Pro Ala Glu Gly Asp Thr Glu
            240                 245                 250

GAA GAT GAT GGA TTT GTG GAC ATC CTA GAG AGT GAC TTA AAG GAT GAT        876
Glu Asp Asp Gly Phe Val Asp Ile Leu Glu Ser Asp Leu Lys Asp Asp
        255                 260                 265

GAT GCA GTT CCC CCA GGC ATG GAG AGT CTC ATT AGT GCC CCA CTG GTC        924
Asp Ala Val Pro Pro Gly Met Glu Ser Leu Ile Ser Ala Pro Leu Val
    270                 275                 280

AAG ACC TTG GAA AAG GAA GAG GAA AAG GAC CTC GTC ATG TAC AGC AAG        972
Lys Thr Leu Glu Lys Glu Glu Glu Lys Asp Leu Val Met Tyr Ser Lys
285                 290                 295                 300

TGC CAG CGG CTC TTC CGC TCT CCG TCC ATG CCC TGC AGC GTG ATC CGG       1020
Cys Gln Arg Leu Phe Arg Ser Pro Ser Met Pro Cys Ser Val Ile Arg
                305                 310                 315

CCC ATC CTC AAG AGG CTG GAG CGG CCC CAG GAC AGG GAC ACG CCC GTG       1068
Pro Ile Leu Lys Arg Leu Glu Arg Pro Gln Asp Arg Asp Thr Pro Val
            320                 325                 330

CAG AAT AAG CGG AGG CGG AGC GTG ACC CCT CCT GAG GAG CAG CAG GAG       1116
Gln Asn Lys Arg Arg Arg Ser Val Thr Pro Pro Glu Glu Gln Gln Glu
        335                 340                 345

GCT GAG GAA CCT AAA GCC CGC GTC CTC CGC TCA AAA TCA CTG TGT CAC       1164
Ala Glu Glu Pro Lys Ala Arg Val Leu Arg Ser Lys Ser Leu Cys His
    350                 355                 360

GAT GAG ATC GAG AAC CTC CTG GAC AGT GAC CAC CGA GAG CTG ATT GGA       1212
Asp Glu Ile Glu Asn Leu Leu Asp Ser Asp His Arg Glu Leu Ile Gly
365                 370                 375                 380

GAT TAC TCT AAG GCC TTC CTC CTA CAG ACA GTA GAC GGA AAG CAC CAA       1260
Asp Tyr Ser Lys Ala Phe Leu Leu Gln Thr Val Asp Gly Lys His Gln
                385                 390                 395

GAC CTC AAG TAC ATC TCA CCA GAA ACG ATG GTG GCC CTA TTG ACG GGC       1308
Asp Leu Lys Tyr Ile Ser Pro Glu Thr Met Val Ala Leu Leu Thr Gly
            400                 405                 410

AAG TTC AGC AAC ATC GTG GAT AAG TTT GTG ATT GTA GAC TGC AGA TAC       1356
Lys Phe Ser Asn Ile Val Asp Lys Phe Val Ile Val Asp Cys Arg Tyr
        415                 420                 425

CCC TAT GAA TAT GAA GGC GGG CAC ATC AAG ACT GCG GTG AAC TTG CCC       1404
Pro Tyr Glu Tyr Glu Gly Gly His Ile Lys Thr Ala Val Asn Leu Pro
    430                 435                 440

CTG GAA CGC GAC GCC GAG AGC TTC CTA CTG AAG AGC CCC ATC GCG CCC       1452
Leu Glu Arg Asp Ala Glu Ser Phe Leu Leu Lys Ser Pro Ile Ala Pro
445                 450                 455                 460

TGT AGC CTG GAC AAG AGA GTC ATC CTC ATT TTC CAC TGT GAA TTC TCA       1500
Cys Ser Leu Asp Lys Arg Val Ile Leu Ile Phe His Cys Glu Phe Ser
                465                 470                 475
```

Figure 1(e) - Panel B

```
TCT GAG CGT GGG CCC CGC ATG TGC CGT TTC ATC AGG GAA CGA GAC CGT         1548
Ser Glu Arg Gly Pro Arg Met Cys Arg Phe Ile Arg Glu Arg Asp Arg
            480                 485                 490

GCT GTC AAC GAC TAC CCC AGC CTC TAC TAC CCT GAG ATG TAT ATC CTG         1596
Ala Val Asn Asp Tyr Pro Ser Leu Tyr Tyr Pro Glu Met Tyr Ile Leu
        495                 500                 505

AAA GGC GGC TAC AAG GAG TTC TTC CCT CAG CAC CCG AAC TTC TGT GAA         1644
Lys Gly Gly Tyr Lys Glu Phe Phe Pro Gln His Pro Asn Phe Cys Glu
    510                 515                 520

CCC CAG GAC TAC CGG CCC ATG AAC CAC GAG GCC TTC AAG GAT GAG CTA         1692
Pro Gln Asp Tyr Arg Pro Met Asn His Glu Ala Phe Lys Asp Glu Leu
525                 530                 535                 540

AAG ACC TTC CGC CTC AAG ACT CGC AGC TGG GCT GGG GAG CGG AGC CGG         1740
Lys Thr Phe Arg Leu Lys Thr Arg Ser Trp Ala Gly Glu Arg Ser Arg
                545                 550                 555

CGG GAG CTC TGT AGC CGG CTG CAG GAC CAG TGAGGGGCCT GCGCCAGTCC           1790
Arg Glu Leu Cys Ser Arg Leu Gln Asp Gln
            560                 565

TGCTACCTCC CTTGCCTTTC GAGGCCTGAA GCCAGCTGCC CTATGGGCCT GCCGGGCTGA       1850

GGGCCTGCTG GAGGCCTCAG GTGCTGTCCA TGGGAAAGAT GGTGTGGTGT CCTGCCTGTC       1910

TGCCCCAGCC CAGATTCCCC TGTGTCATCC CATCATTTTC CATATCCTGG TGCCCCCCAC       1970

CCCTGGAAGA GCCCAGTCTG TTGAGTTAGT TAAGTTGGGT TAATACCAGC TTAAAGGCAG       2030

TATTTTGTGT CCTCCAGGAG CTTCTTGTTT CCTTGTTAGG GTTAACCCTT CATCTTCCTG       2090

TGTCCTGAAA CGCTCCTTTG TGTGTGTGTC AGCTGAGGCT GGGGAGAGCC GTGGTCCCTG       2150

AGGATGGGTC AGAGCTAAAC TCCTTCCTGG CCTGAGAGTC AGCTCTCTGC CCTGTGTACT       2210

TCCCGGGCCA GGGCTGCCCC TAATCTCTGT AGGAACCGTG GTATGTCTGC CATGTTGCCC       2270

CTTTCTCTTT TCCCCTTTCC TGTCCCACCA TACGAGCACC TCCAGCCTGA ACAGAAGCTC       2330

TTACTCTTTC CTATTTCAGT GTTACCTGTG TGCTTGGTCT GTTTGACTTT ACGCCCATCT       2390

CAGGACACTT CCGTAGACTG TTTAGGTTCC CCTGTCAAAT ATCAGTTACC CACTCGGTCC       2450

CAGTTTTGTT GCCCCAGAAA GGGATGTTAT TATCCTTGGG GGCTCCCAGG GCAAGGGTTA       2510

AGGCCTGAAT CATGAGCCTG CTGGAAGCCC AGCCCCTACT GCTGTGAACC CTGGGGCCTG       2570

ACTGCTCAGA ACTTGCTGCT GTCTTGTTGC GGATGGATGG AAGGTTGGAT GGATGGGTGG       2630

ATGGCCGTGG ATGGCCGTGG ATGCGCAGTG CCTTGCATAC CCAAACCAGG TGGGAGCCTT       2690

TTGTTGAGCA TGACACCTGC AGCAGGAATA TATGTGTGCC TATTTGTGTG GACAAAAATA       2750

TTTACACTTA GGGTTTGGAG CTATTCAAGA GGAAATGTCA CAGAAGCAGC TAAACCAAGG       2810

ACTGAGCACC CTCTGGATTC TGAATCTCAA GATGGGGGCA GGGCTGTGCT TGAAGGCCCT       2870

GCTGAGTCAT CTGTTAGGGC CTTGGTTCAA TAAAGCACTG AGCAAGTTGA GAAAAAAAAA       2930

AAAAAAAAAA                                                              2940
```

Figure 1(f) - Panel B

```
cdc25A  1-318  LDNDPRDLIGDESKGYLFHTVAGKHQDLKYISPEIMASVLNGKFANLIKEFVIIDCRYPYEYEGGHIKGAVNLHMEEE
cdc25B  1-361  LDSDHRELIGDYSKAFLLQTVDGKHQDLKYISPETMVALLTGKFSNIVDKFVIVDCRYPYEYEGGHIKTAVNLPLERD
cdc25C  1-276  EDSNQGHLIGDFSKVCALPTVSGKHQDLKYVNPETVAALLSGKFQGLIEKFYVIDCRYPYEYLGGHIQGALNLYSQEE
stg     1-269  ENRNEPELIGDESKAYSLPLMEGRHRDLKSISSETVARLLKGEFSDKVASYRIIDCRYPYEFEGGHIEGAKNLYTTEQ
25Sp    1-382  STKESERFISSHVEDLSLPCFAVKEDSLKRITQETLLGLLDGKFKDIFDKCIIIDCRFEYEYLGGHISTAVNLNTKQA

cdc25A         VEDFLLKKPIVP--------TDGKRVIVVFHCEFSSERGPRMCRYVRERDRLGNE--YPKLHYPELYVLKGGYKEFFM
cdc25B         AESFLLKSPIAPC--------SLDKRVILIFHCEFSSERGPRMCRFIRERDRAVND--YPSLYYPEMYILKGGYKEFFP
cdc25C         LFNEELKKPIVPL--------DTQKRIIIVFHCEFSSERGPRMCRCLREEDRSLNQ--YPALYYPELYILKGGYRDFFP
stg            ILDEFLTVQQTELQQQQNAESGHKPNIIIFHCEFSSERGPKMSRGLRNLDRERNTNAYPALHYPEIYLLHNGYKEFFE
25Sp           IVDAEISKPLT-----------HVRA-LVFHCEHSAHRAPHLALHFRNTDRRMNSHRYPFLYYPEMYILHGGYKSFYE

cdc25A         KCQSYCEPPSYRPMHHEDFKEDLKKFRTKSRTWAGEKSKREMYSRLKKL  523
cdc25B         QHPNFCEPQDYRPMNHEAFKDELKTFRLKTRSWAGERSRRELCSRLQDQ  566
cdc25C         EYMELCEPQSYCPMHHQDHKTELLRCRSQSKVQEGERQLREQIALLVKDMSP  474
stg            SHVELCEPHAYRTMLDPAYNEAYRHFRAKSKS-WNGDGLGGATGRLKKSRSRLML  479
25Sp           NHKNRCDPINYVPMNDRSHVNTCTKAMNNFKR-NATFMRTKSYTFWPKCVSFPRR  580
```

Figure 2

Figure 3(a)

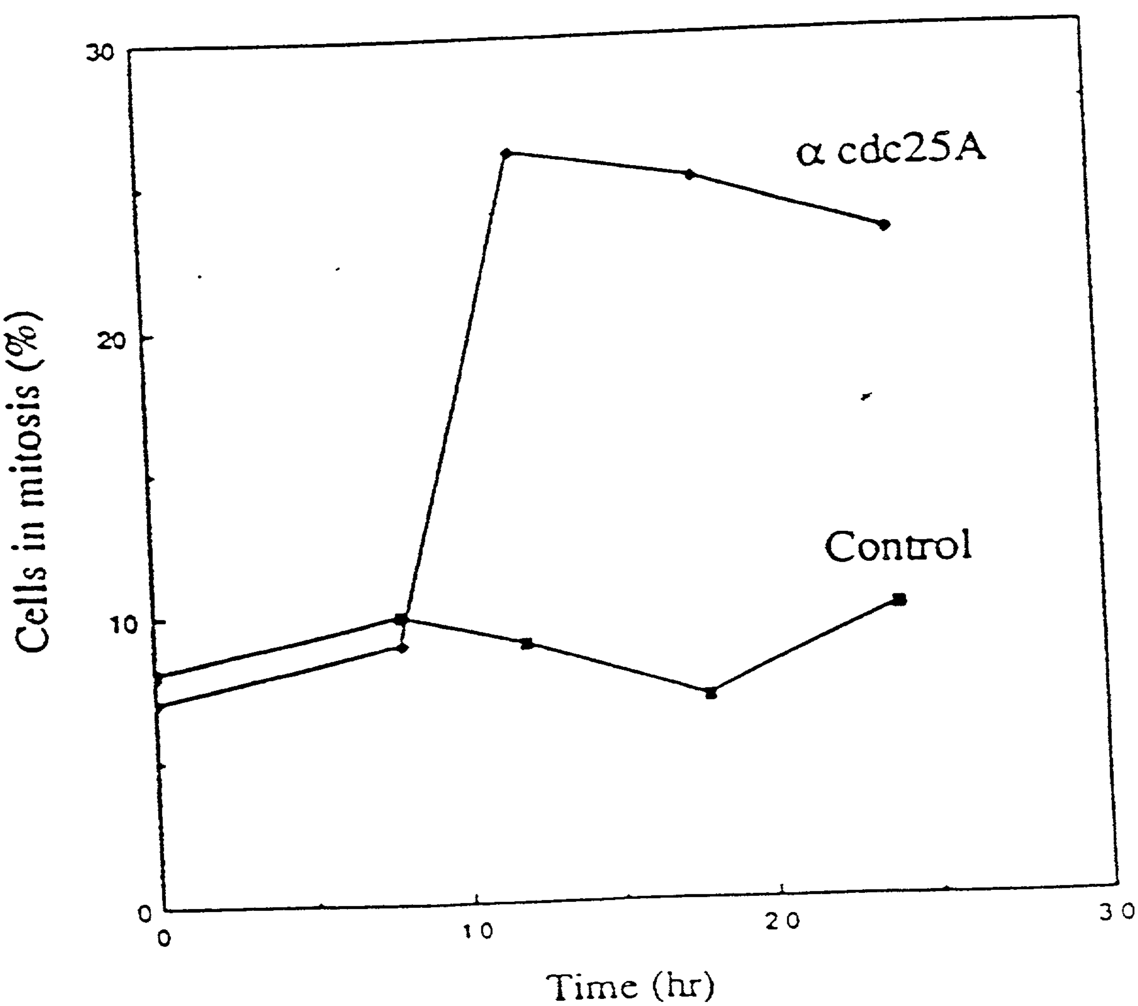
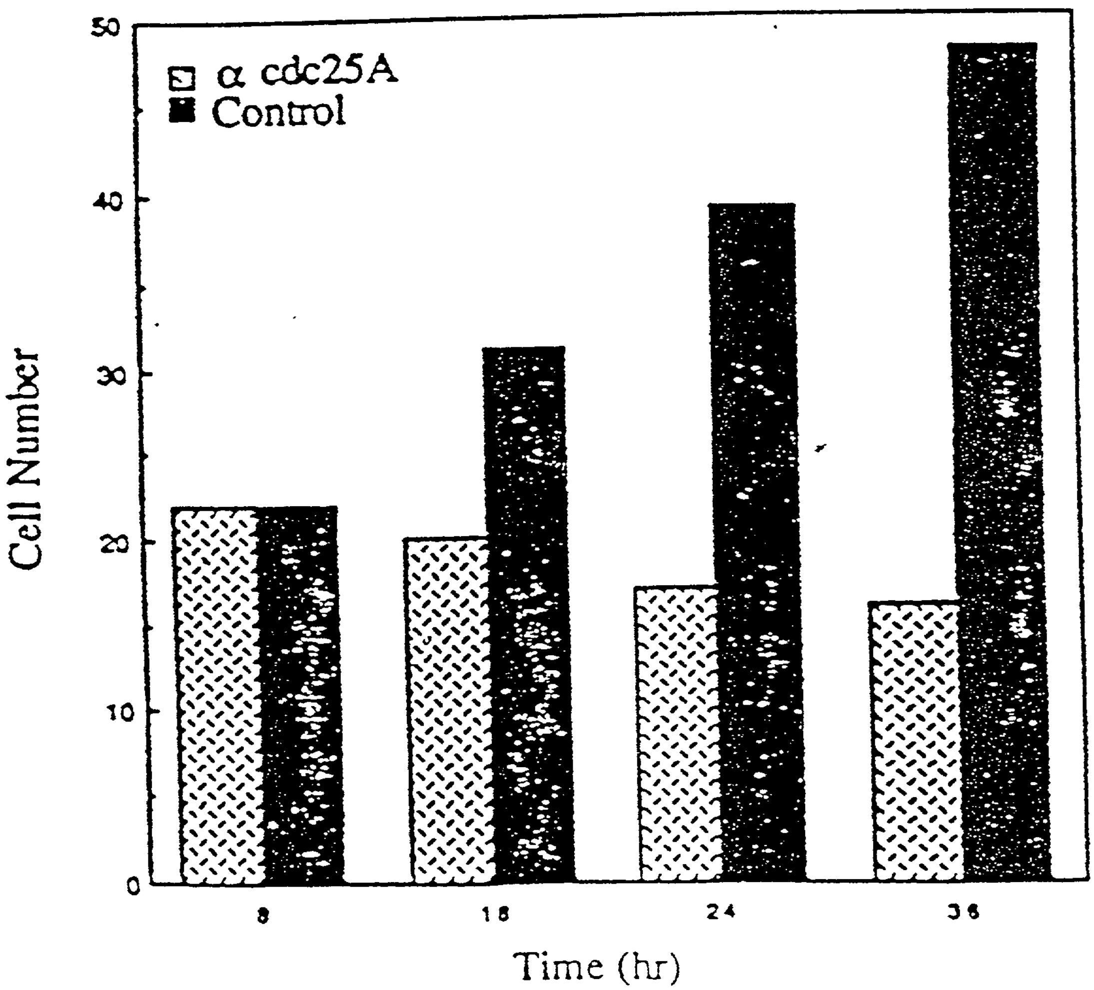


Figure 3(b)



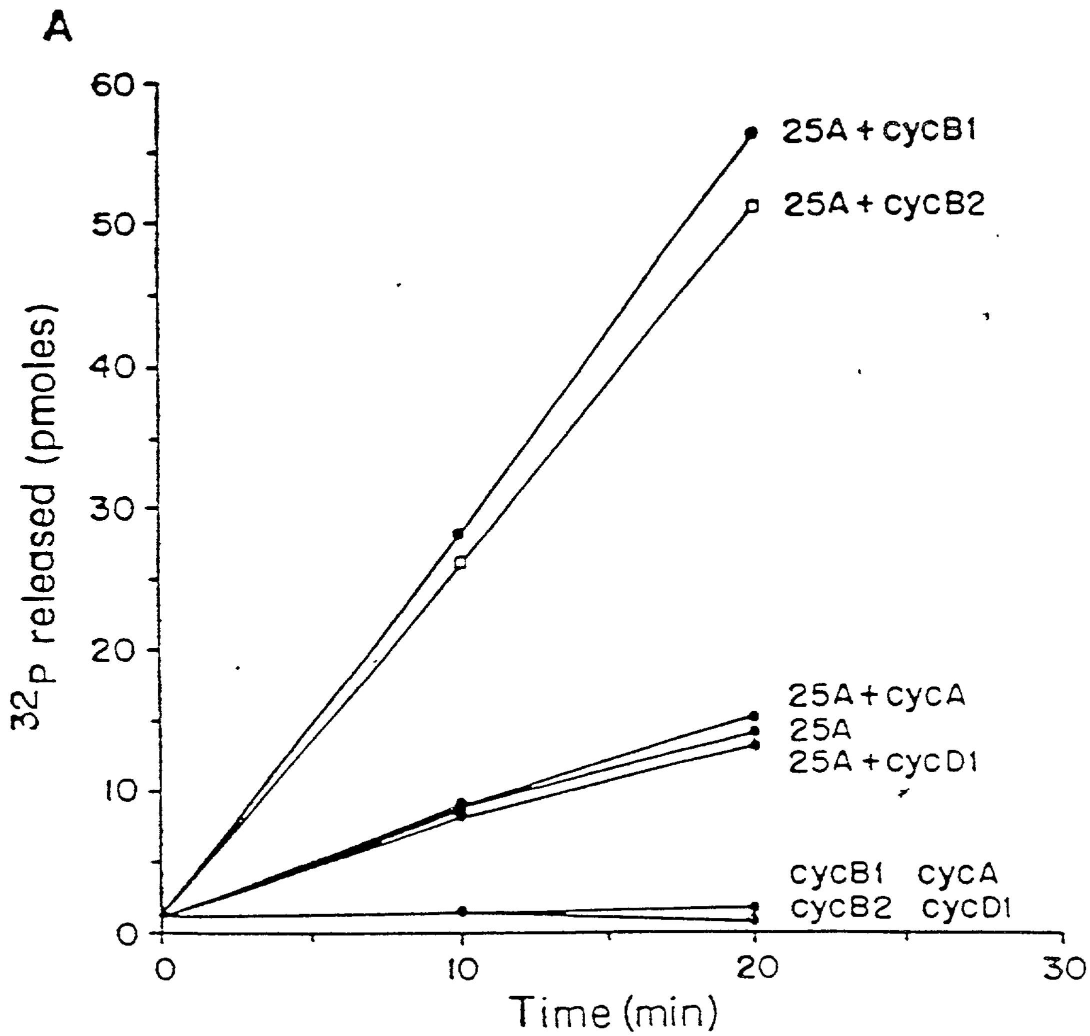

A
60
50
40
30
20
10
0
^{32}P released (pmoles)
25A + cycB1
25A + cycB2
25A + cycA
25A
25A + cycD1
cycB1 cycA
cycB2 cycD1
0
10
20
30
Time (min)

Figure 4(a)

B

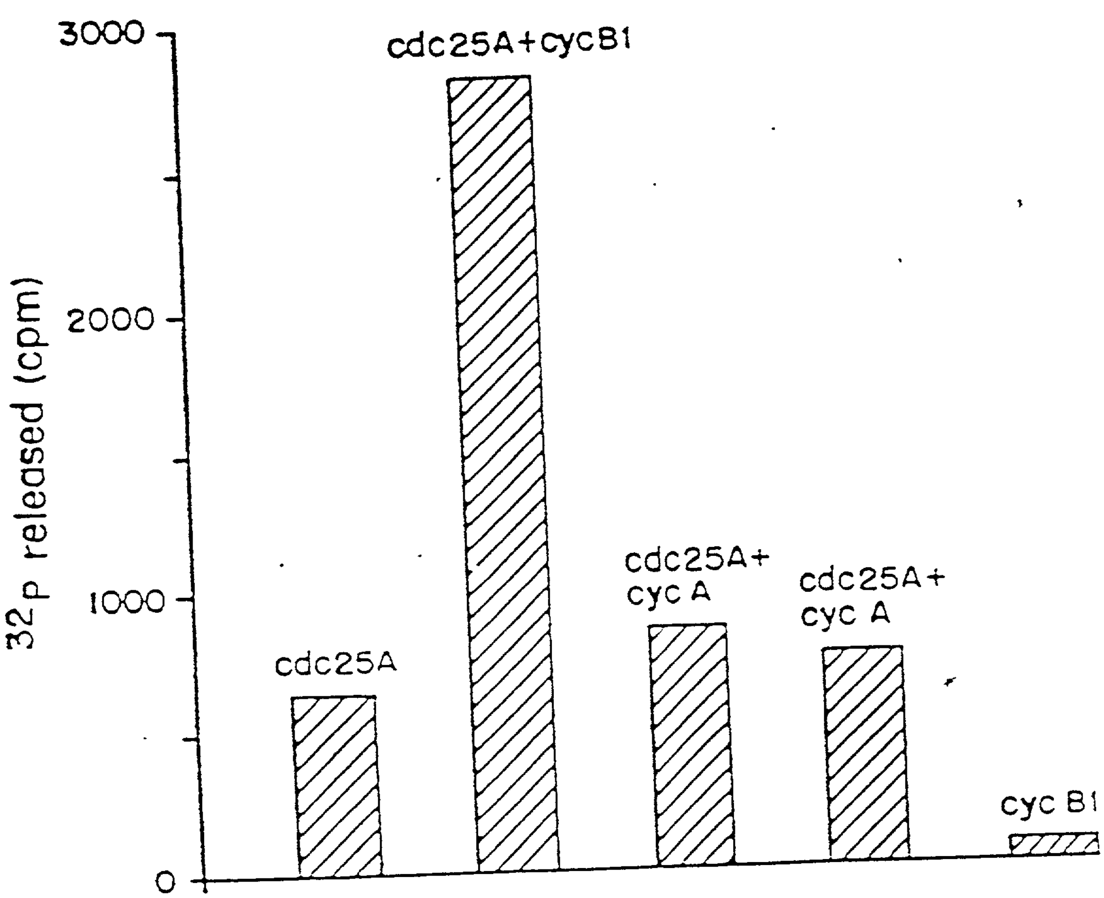

3000
2000
1000
0
32p released (cpm)
cdc25A+cycB1
cdc25A
cdc25A+
cyc A
cdc25A+
cyc A
cyc B1

Figure 4(b)

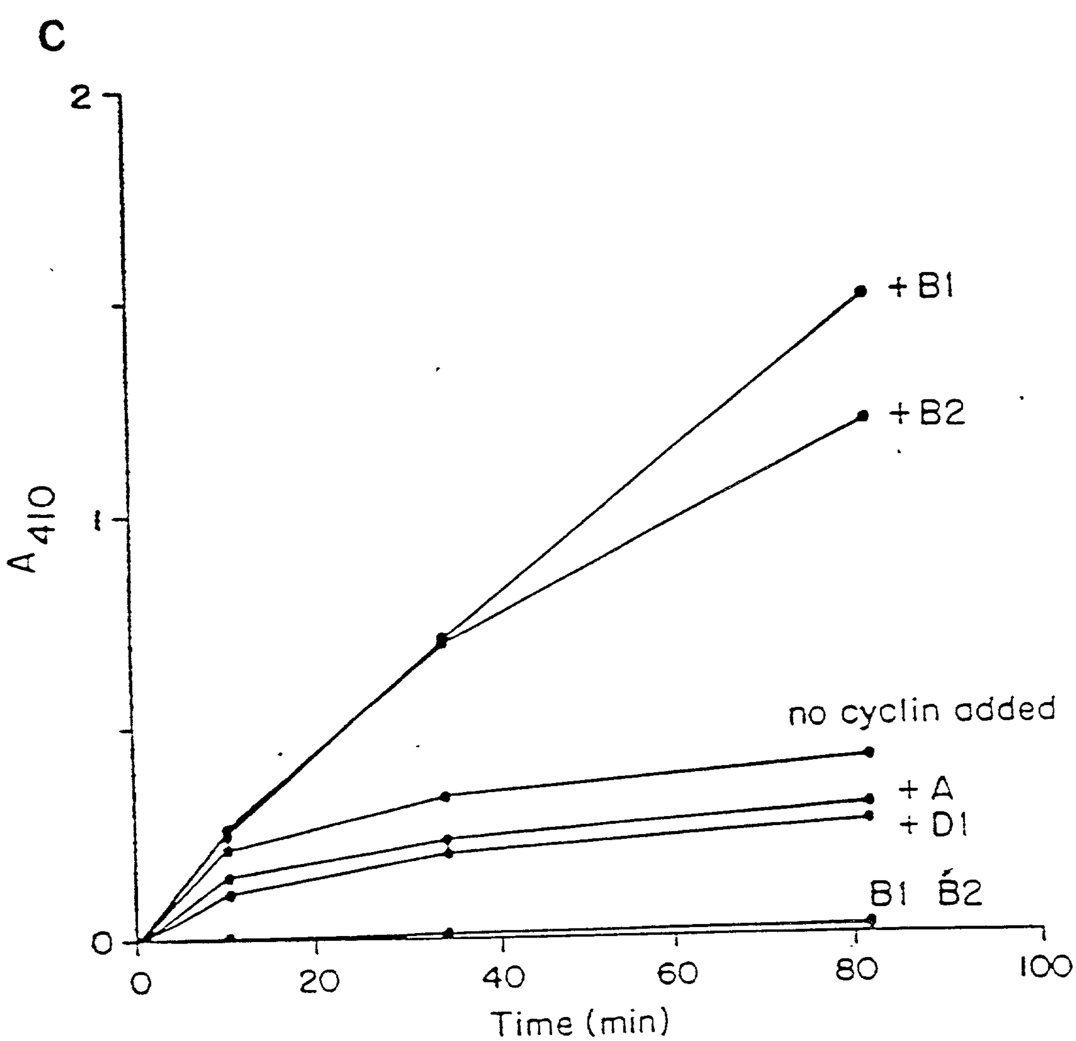

C
2
1
0
A_{410}
+B1
+B2
no cyclin added
+A
+D1
B1 B2
0
20
40
60
80
100
Time (min)

Figure 4(c)

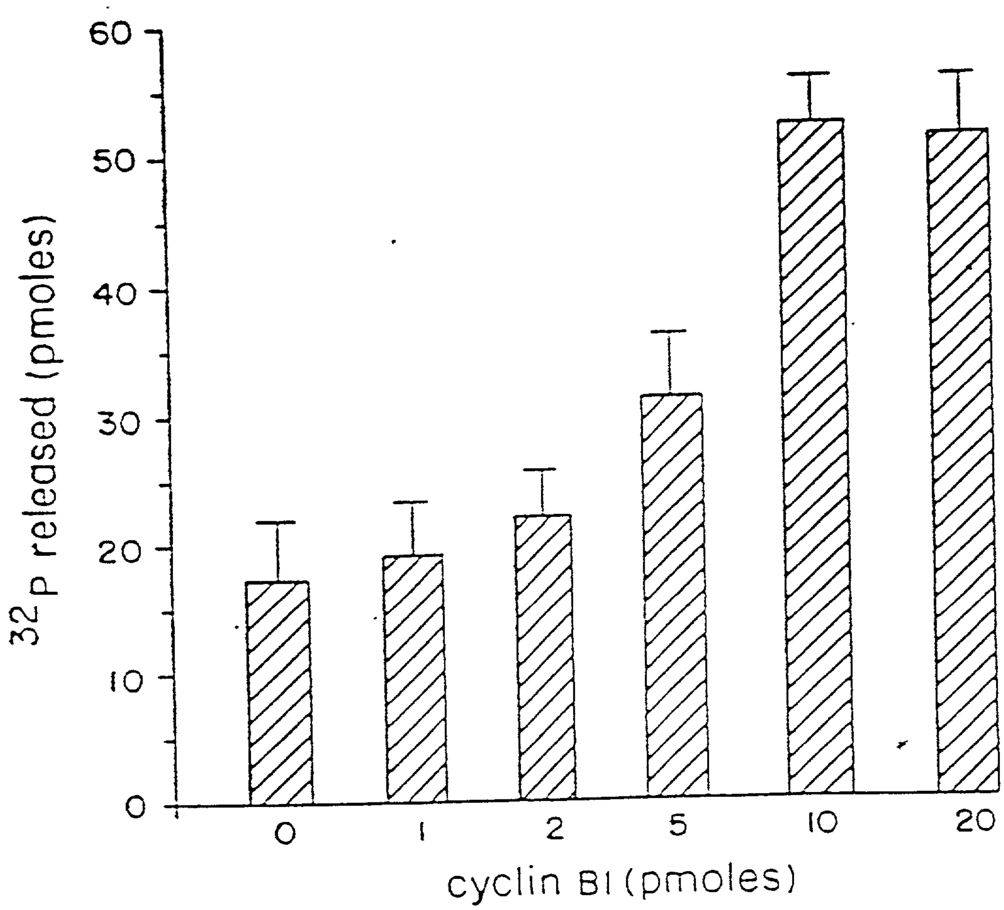
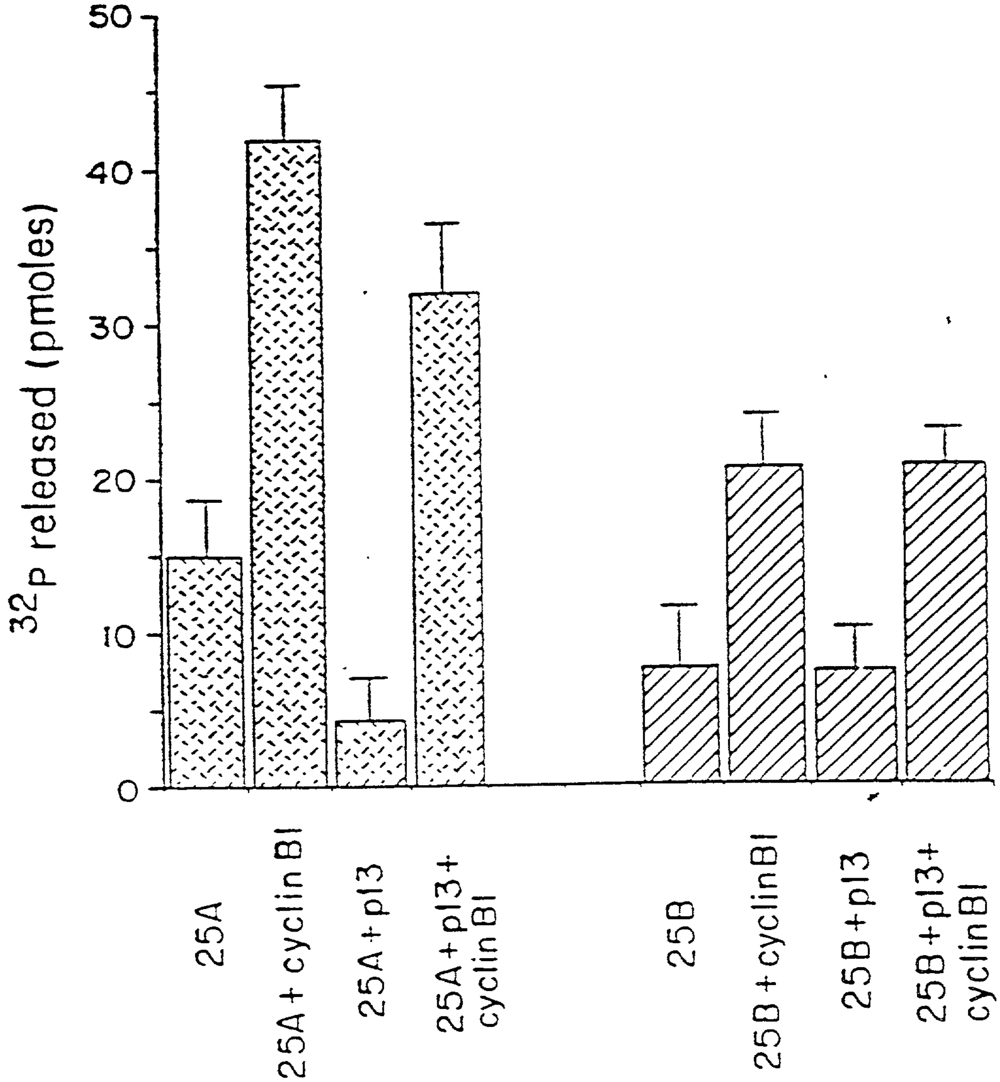

60
50
40
30
20
10
0
^{32}P released (pmoles)
0
1
2
5
10
20
cyclin B1 (pmoles)

Figure 5



50
40
30
20
10
0
32p released (pmoles)
25A
25A + cyclin BI
25A + pl3
25A + pl3 + cyclin BI
25B
25B + cyclin BI
25B + pl3
25B + pl3 + cyclin BI

Figure 6

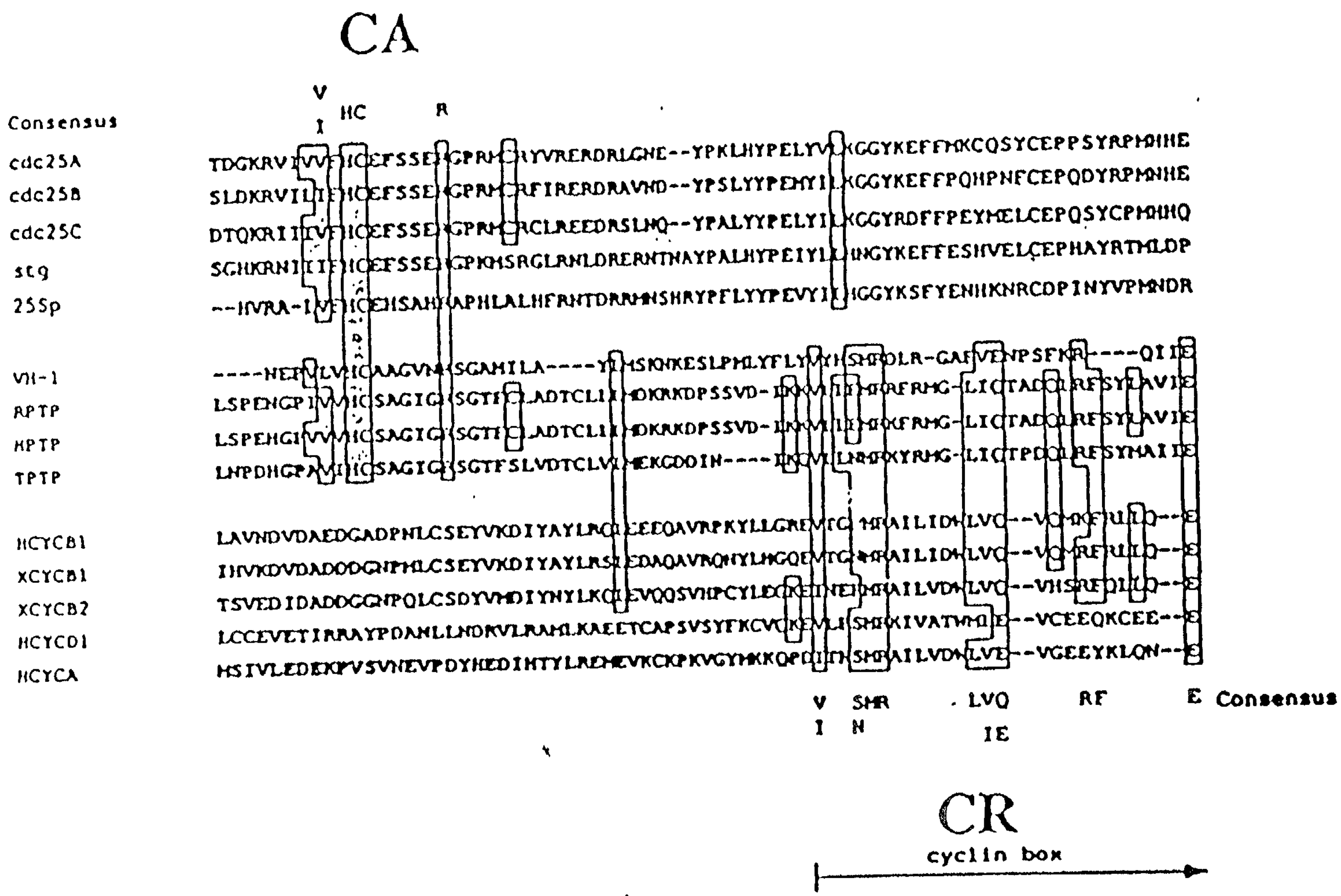

CA
Consensus
cdc25A
cdc25B
cdc25C
stg
25Sp
VH-1
RPTP
HPTP
TPTP
HCYCB1
XCYCB1
XCYCB2
HCYCD1
HCYCA
CR
cyclin box

Figure 7(a)

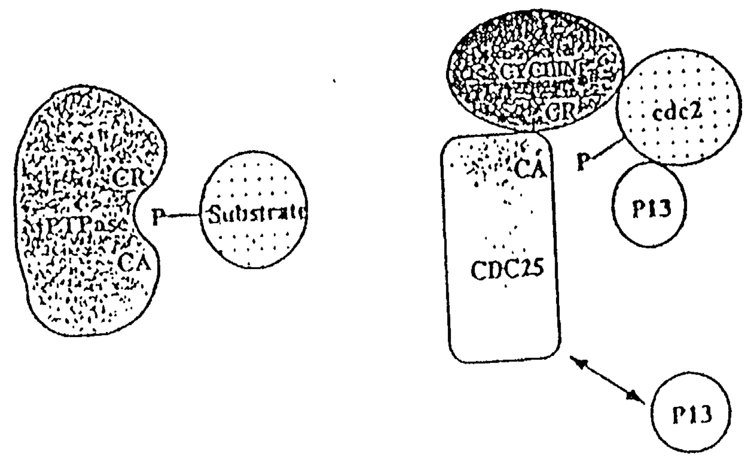
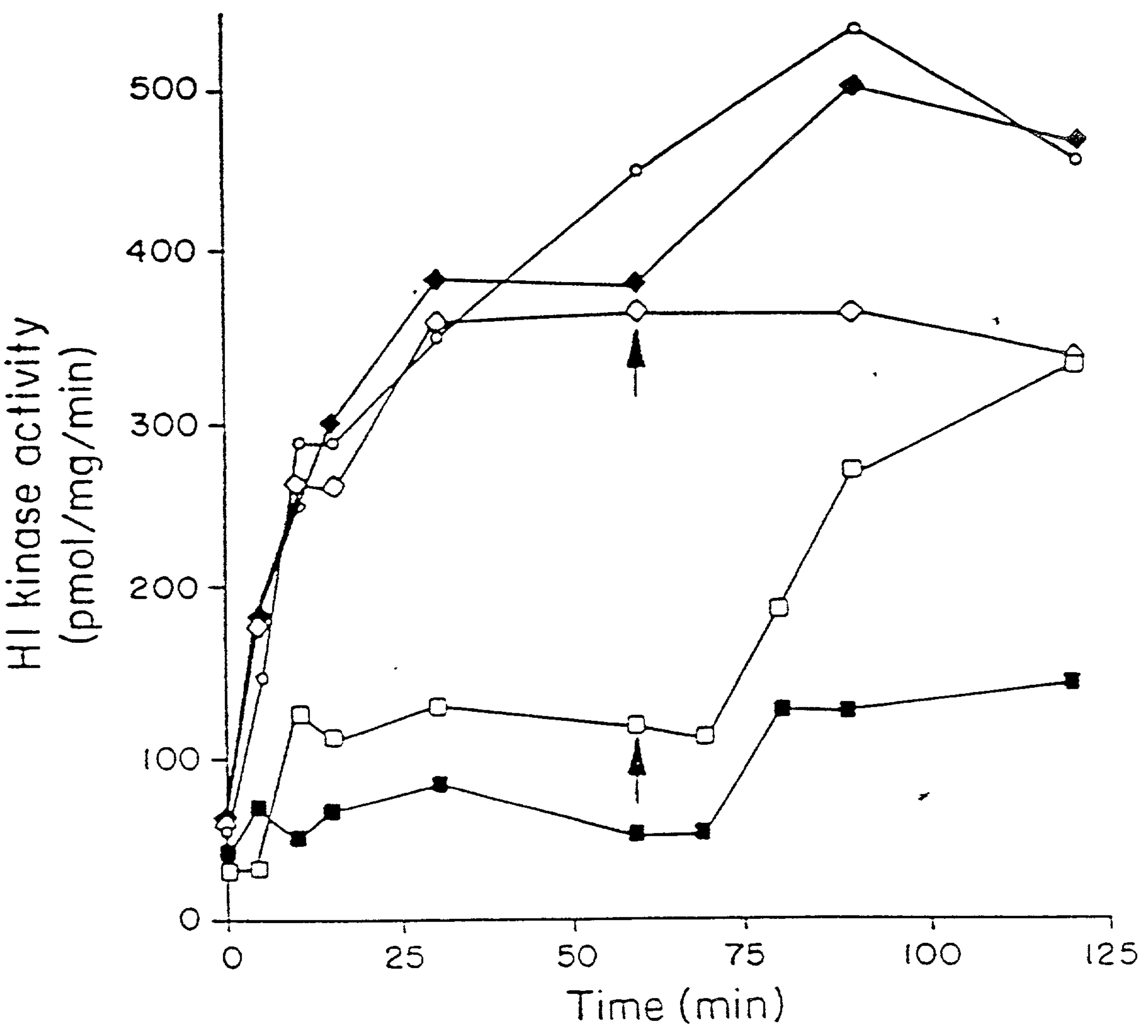


Figure 7(b)



H1 kinase activity (pmol/mg/min)
500
400
300
200
100
0
0
25
50
75
100
125
Time (min)

Figure 8

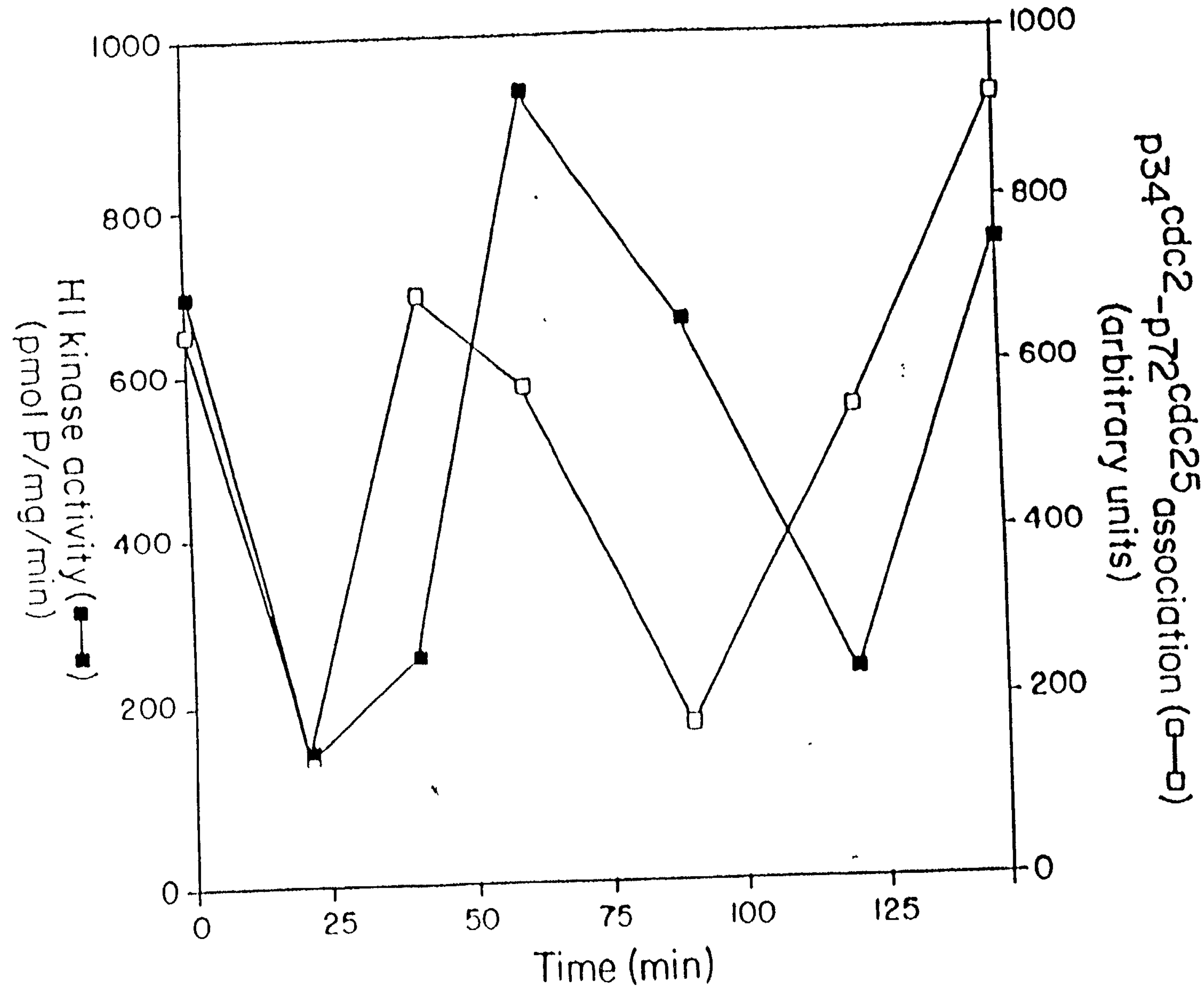

HI kinase activity
(pmol P/mg/min)
$p34^{cdc2}$-$p72^{cdc25}$ association
(arbitrary units)
Time (min)
0
25
50
75
100
125
200
400
600
800
1000

Figure 9

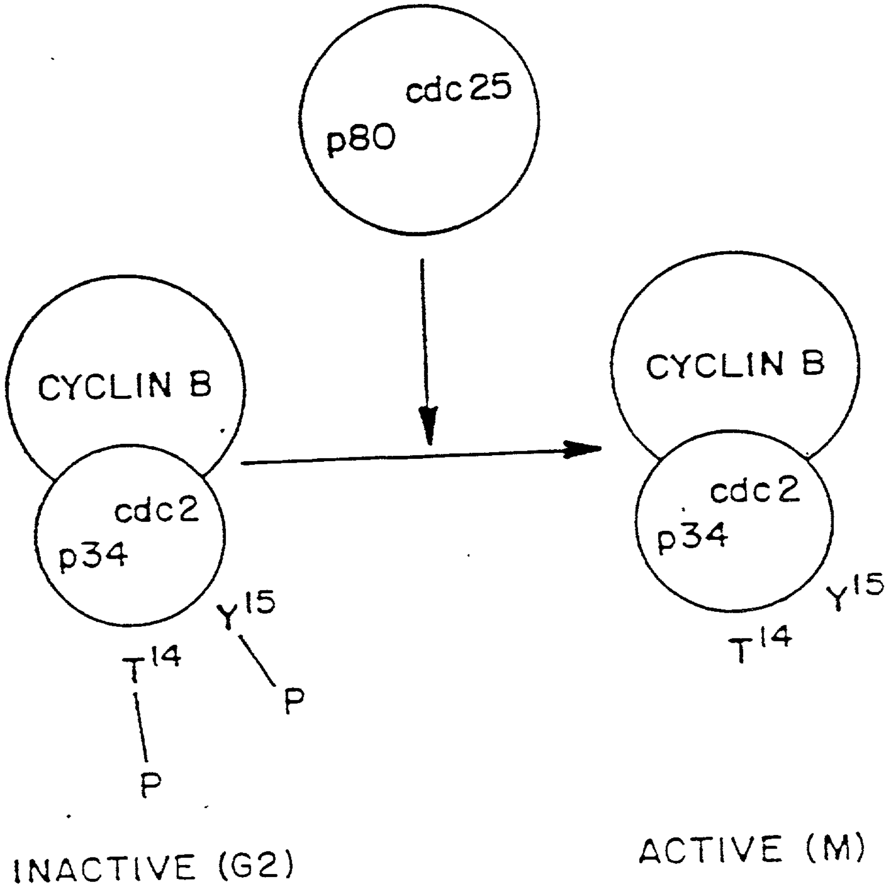

cdc25
p80
CYCLIN B
cdc2
p34
Y^{15}
T^{14}
P
P
INACTIVE (G2)
CYCLIN B
cdc2
p34
Y^{15}
T^{14}
ACTIVE (M)

Figure 10

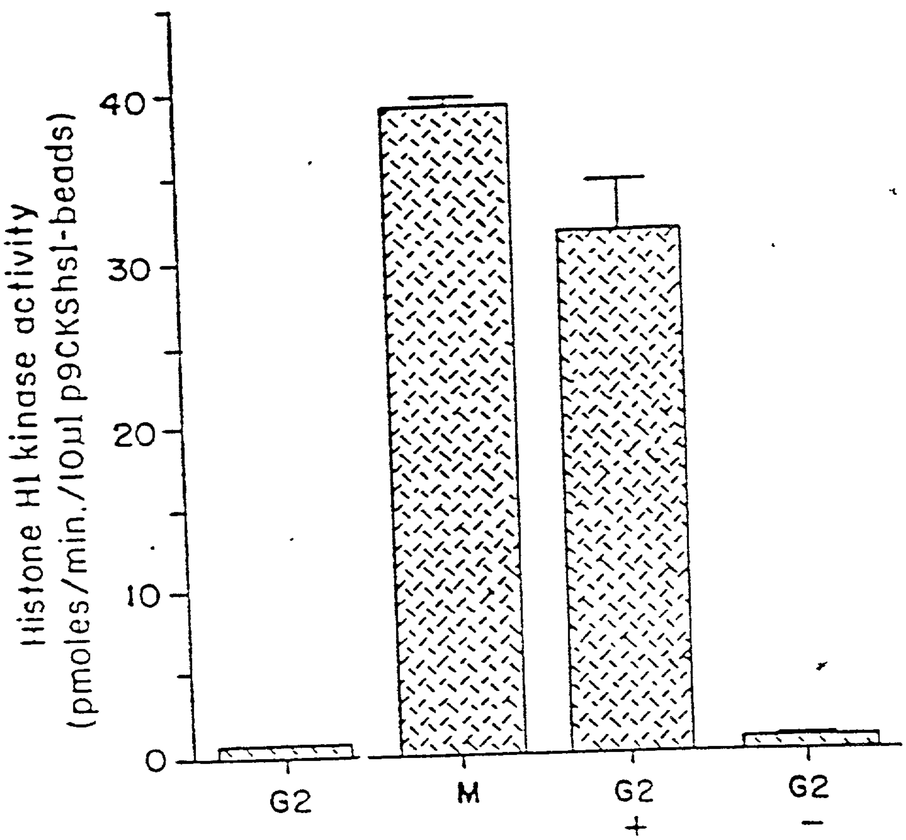

Histone H1 kinase activity
(pmoles/min./10µl p9CKShs1-beads)
40
30
20
10
0
G2
M
G2
+
G2
−

Figure 11

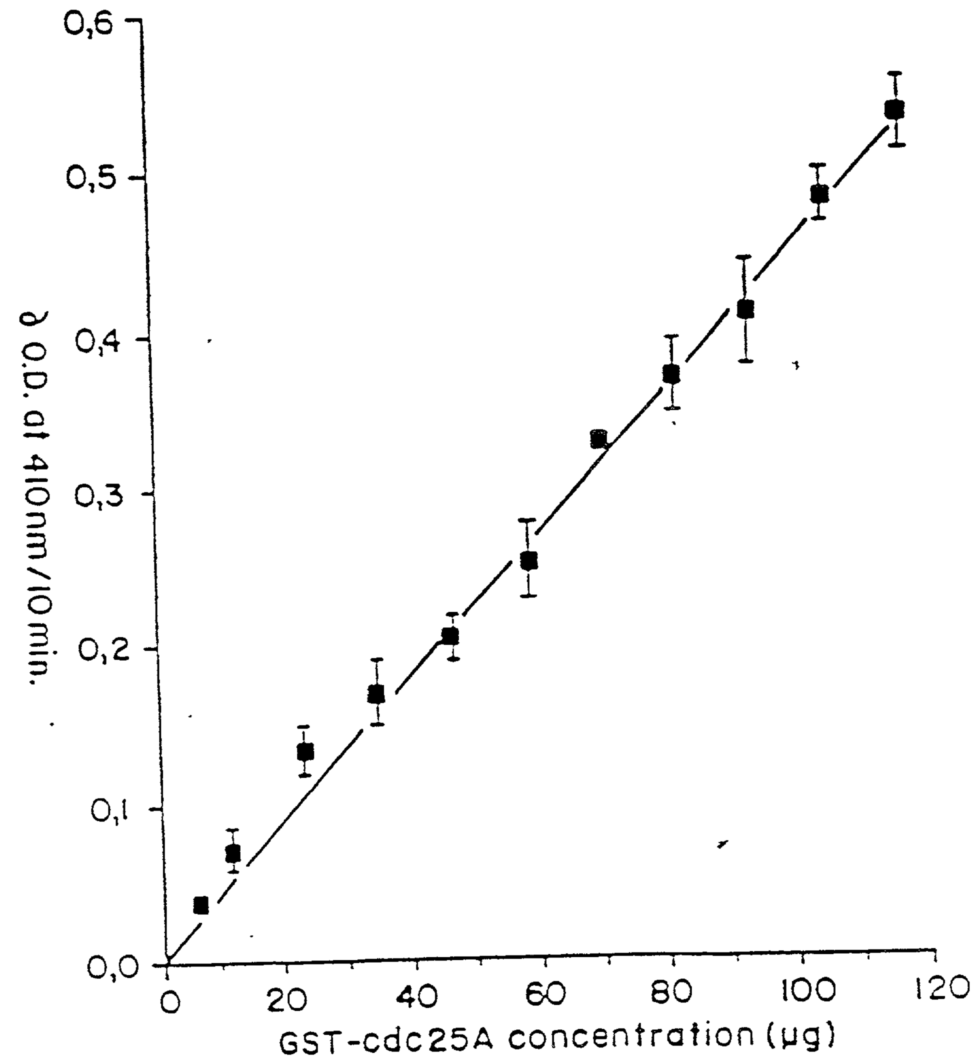

0,6
0,5
0,4
0,3
0,2
0,1
0,0
∂ O.D. at 410nm/10min.
0
20
40
60
80
100
120
GST-cdc25A concentration (µg)

Figure 12(a)

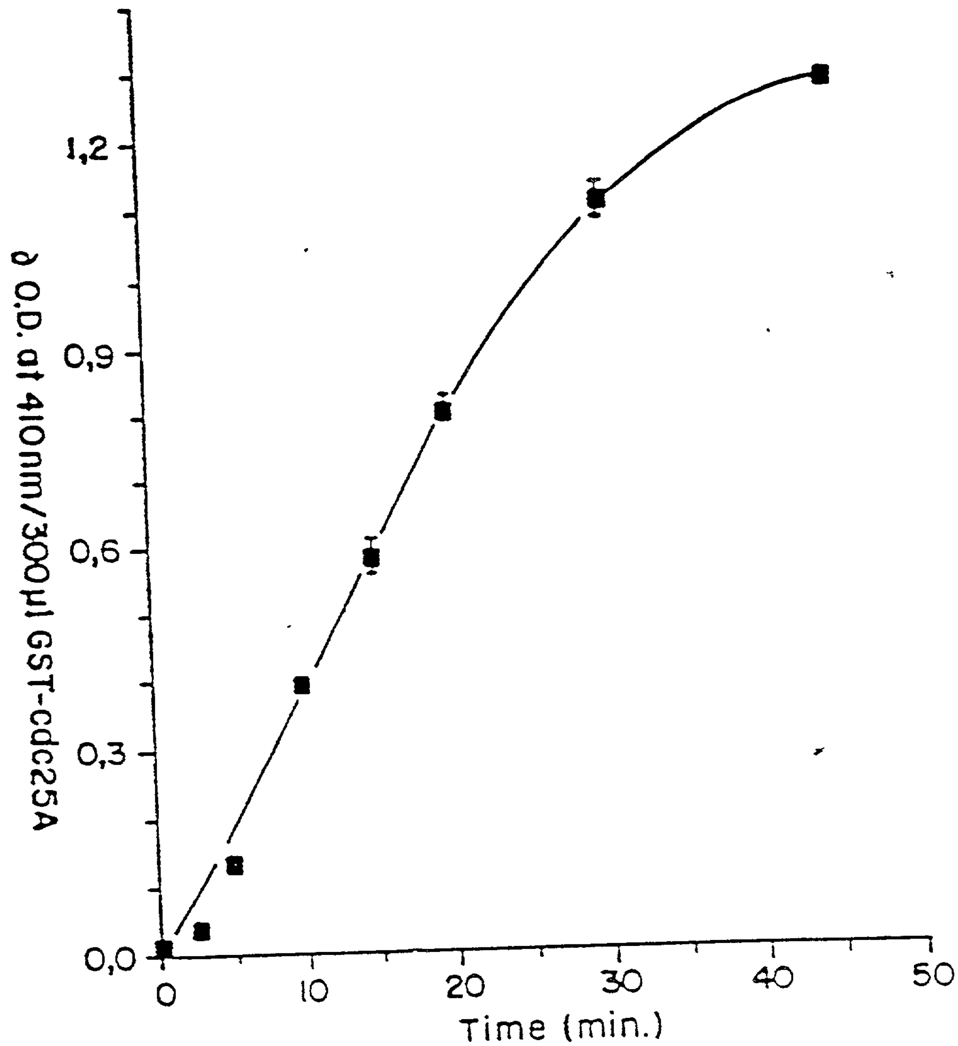
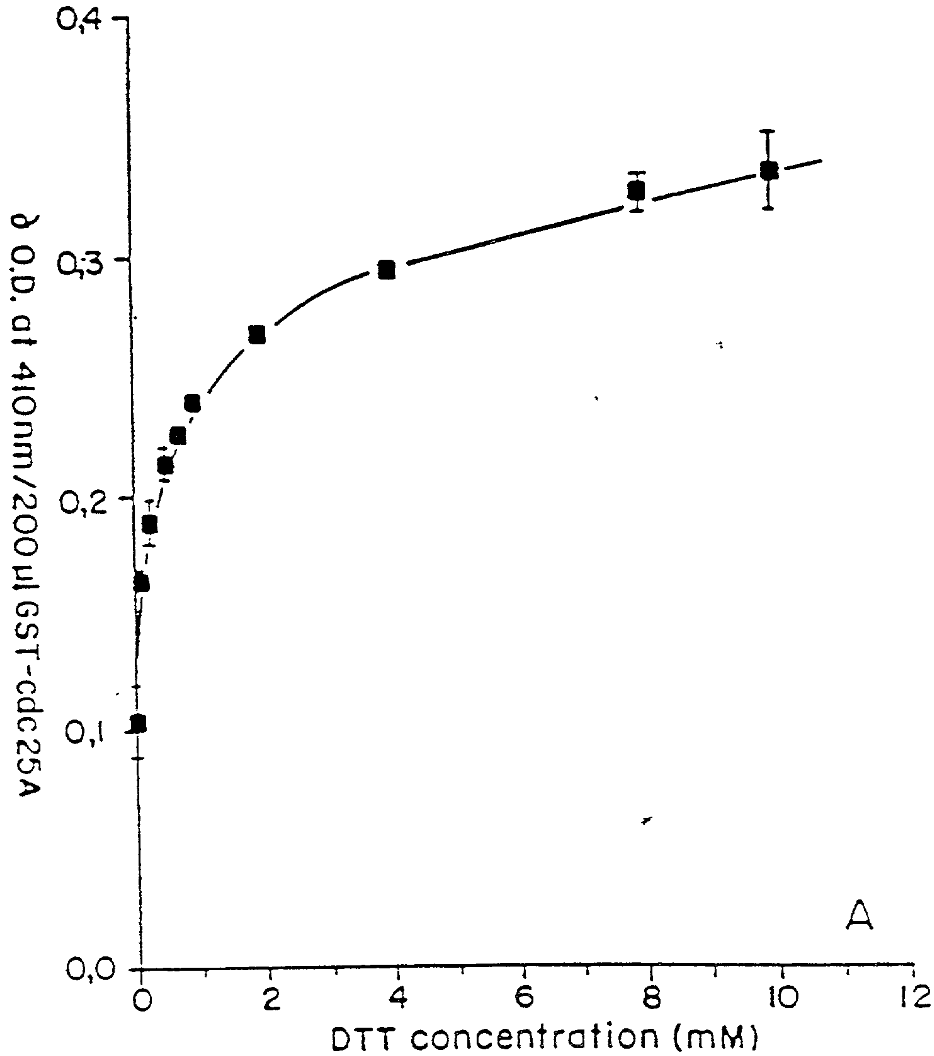

∂ O.D. at 410nm/300µl GST-cdc25A
1,2
0,9
0,6
0,3
0,0
0
10
20
30
40
50
Time (min.)

Figure 12(b)



0,4
0,3
0,2
0,1
0,0
ð O.D. at 410nm/200 µl GST-cdc25A
A
0
2
4
6
8
10
12
DTT concentration (mM)

Figure 13(a)

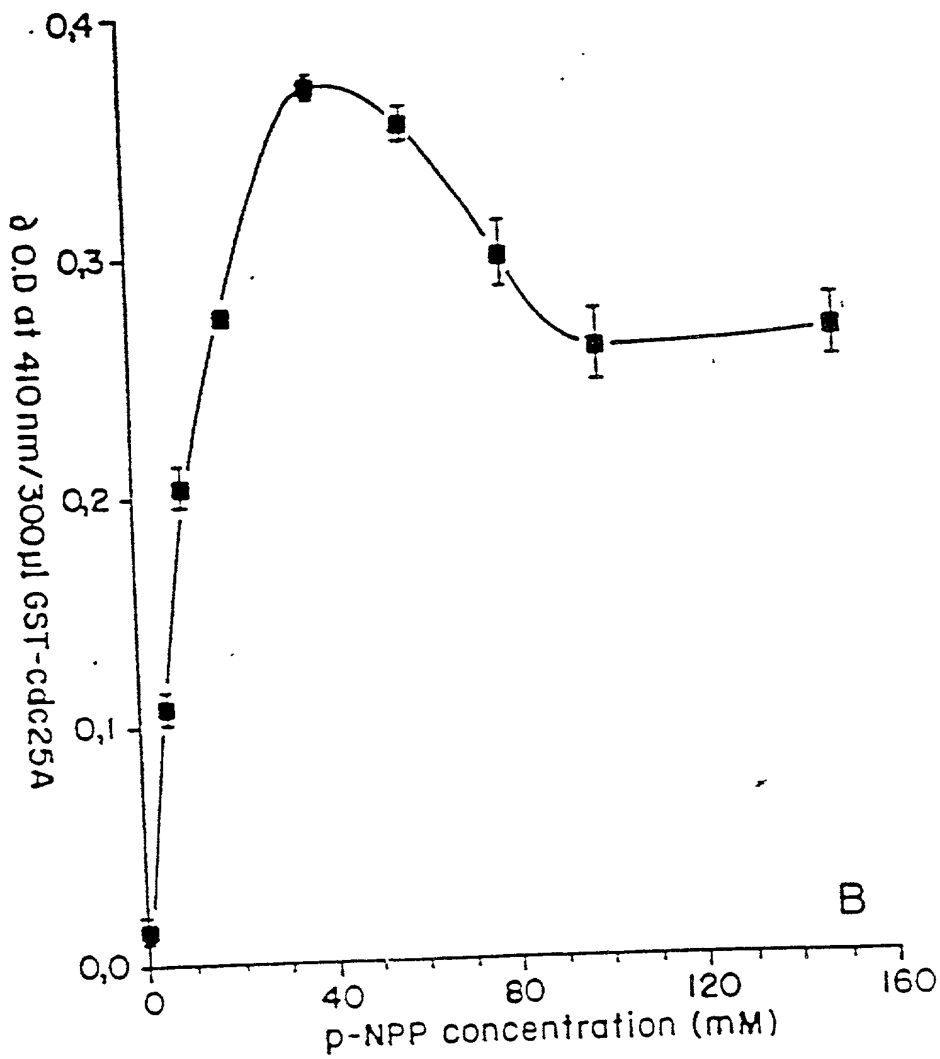
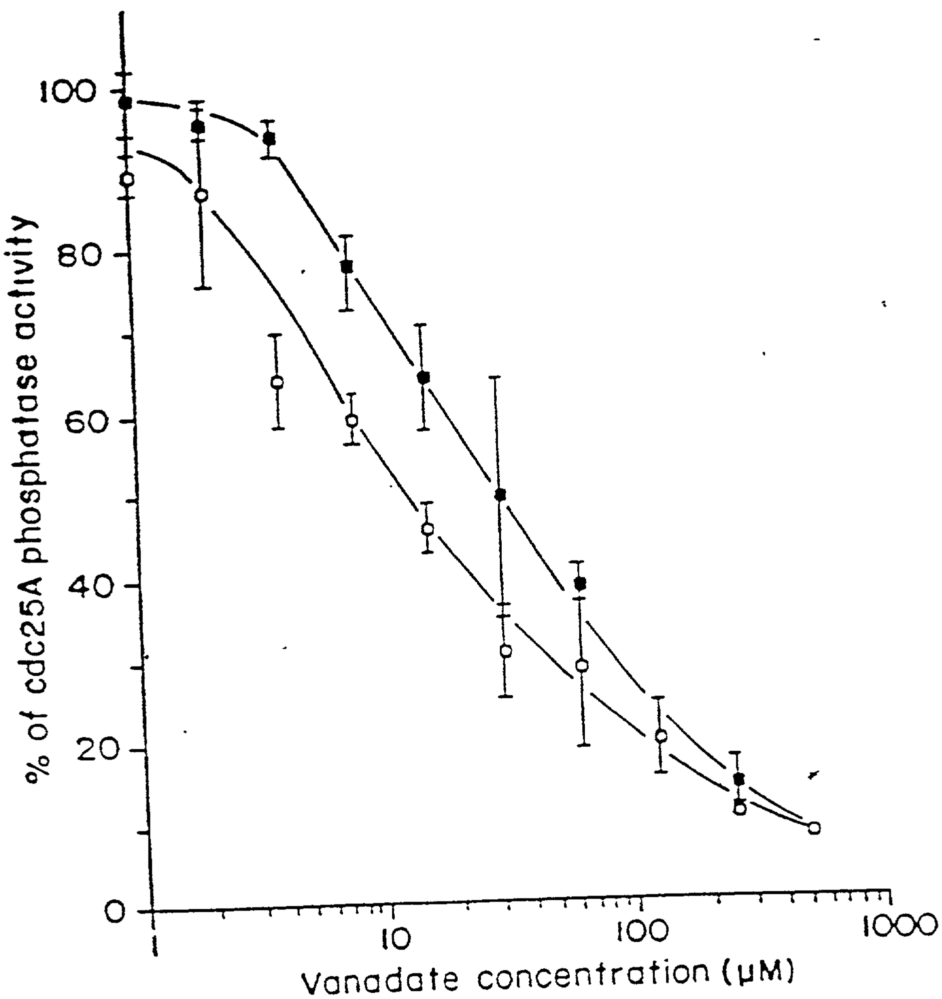

0,4
0,3
0,2
0,1
0,0
∂ O.D at 410nm/300µl GST-cdc25A
B
0
40
80
120
160
p-NPP concentration (mM)

Figure 13(b)



% of cdc25A phosphatase activity
100
80
60
40
20
0
1
10
100
1000
Vanadate concentration (μM)

Figure 14

Attorney's
Docket :MII-019CP3

Declaration, Petition and Power of Attorney For Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

"NOVEL CDC25 GENES, ENCODED PRODUCTS AND USES THEREOF"

the specification of which was filed on April 24, 1995 in the United States Patent and Trademark Office as U.S.S.N. 08/428,415, which is a continuation-in-part of U.S.S.N. 08/379,685 filed January 26, 1995 and entitled *"NOVEL HUMAN CDC25 GENES, ENCODED PRODUCTS AND USES THEREFOR"* which is a continuation-in-part of U.S.S.N. 08/124,569, filed 20 September 1993, which is a continuation-in-part of U.S.S.N. 07/793,601, filed 18 November 1991, and is a continuation-in-part of U.S.S.N. 08/189,206, filed 31 January 1994, which is a continuation-in-part of U.S.S.N. 07/878,640, filed 5 May 1992, and is a continuation-in-part of U.S.S.N. 07/793,601, filed 18 November 1991.

I do not know and do not believe that the subject matter of this application was ever known or used in the United States before my invention thereof or patented or described in any printed publication in any country before my invention thereof or more than one year prior to the date of this application, and that said subject matter has not been patented or made the subject of an issued inventor's certificate in any country foreign to the United States on an application filed by me or my legal representatives or assigns more than twelve months prior to the date of this application; that I acknowledge my duty to disclose information of which I am aware which is material to the examination of this application, that no application for patent or inventor's certificate on the subject matter of this application has been filed by me or my representatives or assigns in any country foreign to the United States, except those identified below, and that I have reviewed and understand the contents of the specification, including the claims as amended by any amendment referred to herein.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

Check one:

_ no such applications have been filed.

X such applications have been filed as follows

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Country	Application Number	Date of Filing (month,day,year)	Priority Claimed Under 35 USC 119
PCT	PCT/US92/10052	November 17, 1992	__ Yes No X
			_ Yes No _
			_ Yes No _
			_ Yes No _
			_ Yes No _

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application. As to subject matter of this application which is common to my earlier United States application, if any, described below, I do not believe that the same was ever known or used in the United States before my invention thereof or patented or described in any printed publication in any country before my invention thereof or more than one year prior to said earlier application, or in public use or on sale in the United States more than one year prior to said earlier application, that the said common subject matter has not been patented or made the subject of an inventor's certificate issued before the date of said earlier application in any country foreign to the United States on an application, filed by me or my legal representatives or assigns more than twelve months prior to said application and that no application for patent or inventor's certificate on said subject matter has been filed by me or my representatives or assigns in any country foreign to the United States except those identified herein.

07/793,601	18 November 1991	Abandoned
(Application Serial No.)	(Filing Date)	(Status)
07/878,640	5 May 1992	Issued, U.S.S.N. 5,294,538
(Application Serial No.)	(Filing Date)	(Status)
08/124,569	20 September 1993	September ~~Pending~~
(Application Serial No.)	(Filing Date)	(Status)
08/189,206	31 January 1994	Pending
(Application Serial No.)	(Filing Date)	(Status)
08/379,685	26 January 1995	Pending
(Application Serial No.)	(Filing Date)	(Status)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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Wherefore I petition that letters patent be granted to me for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe my name to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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